Identification of αvβ6-Positive Stem Cells in Oral Squamous Cell Carcinoma

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Abstract. *Oral squamous cell carcinoma (SCC) is composed of a heterogeneous population of cells which range anywhere from epithelial to mesenchymal in phenotype. Several oral cancer specimens with antibodies to TRA160, a marker of pluripotent cells, were screened. Compared with the well differentiated lesions, pluripotent cells were more numerous in specimens from poorly differentiated tumors. In vitro, the expression of TRA160 was much greater in invasive oral SCC9β6 cells compared with the poorly invasive SCC9SN or SCC9β6D1 cells, which express a truncated β6. In vitro, pluripotent cells were instrumental in aggressively closing an experimental wound assay. Lastly, TRA-1-60+/β6+ tumor cells which formed vascular-like structures in vivo were identified. SCC9β6 cells formed interconnecting channels, whereas SCC9SN cells did not in an in vitro Matrigel angiogenesis assay. The results of this study clearly demonstrated the differential distribution of pluripotent stem cells in oral SCC and that the β6 integrin may be an important regulatory component of the pluripotent phenotype.*

Each year 34,000 Americans are diagnosed with oral or pharyngeal cancer and over 8,000 deaths per year can be attributed to this disease. Squamous cell carcinoma (SCC) accounts for over 96% of these tumors and the prognosis for patients with this disease has not improved in over 50 years (1). Oral SCC, like many solid tumors, contains a heterogeneous population of cancer cells ranging in morphology from classical epithelial to mesenchymal. Stem cells have been previously identified in oral SCC lesions (2). Cancer stem cells have also recently been identified in solid tumors of the breast, lung and central nervous system (3). This heterogeneity may account for the fact that not all of the cancer cells in sold tumors have a similar ability to drive tumor formation and may possess a differential sensitivity to radiation and chemotherapeutic drugs (3).

Solid tumor development consists of a series of stages in which cells undergo a genetic and a phenotypic alteration that allows them to evade normal regulation and to finally colonize distant sites in the body (3). Tumor growth is limited by how rapidly the vasculature can develop to provide the growing cancer cell mass with adequate nutrition and removal of toxic waste products (4). But as tumors become larger and enter the exponential growth phase, angiogenesis alone may be insufficient to meet their increasing vascular needs (4). One strategy to enhance the development of a vasculature capable of supporting continued and rapid tumor growth includes the secretion of factors or the production of additional proteinases to increase degradation of the extracellular matrix (4). The development of an adjunct vascular system to enhance the tumor blood network, such as one lined directly by tumor cells that connects and communicates with the endothelial-lined vasculature, is one possibility and is termed vascular mimicry (4).

Emerging data indicates that within the primary tumor there exists a small subset of cells which possess an indefinite capacity for self renewal. These are stem cells, which in theory, may differ substantially from the majority of the cells within the general population (3). The theory suggests that conventional chemotherapies kill differentiated or differentiating cells, which form the bulk of the tumor and these stem cells could present as a source of disease relapse (3).

In this study subpopulations of oral SCC cells that express TRA-1-60, a marker of pluripotency, were identified. This corresponds well to the observation that most cells in a tumor have a limited ability to divide and only a small subset of phenotypically distinct cancer stem cells has the capacity to self renew and from new tumors (5).

Materials and Methods

Cell culture. The SCC9 cell line (derived from a tongue lesion) was obtained from Dr. James Reinwald (Brigham and Woman's Hospital, Harvard School of Medicine, USA) and has been

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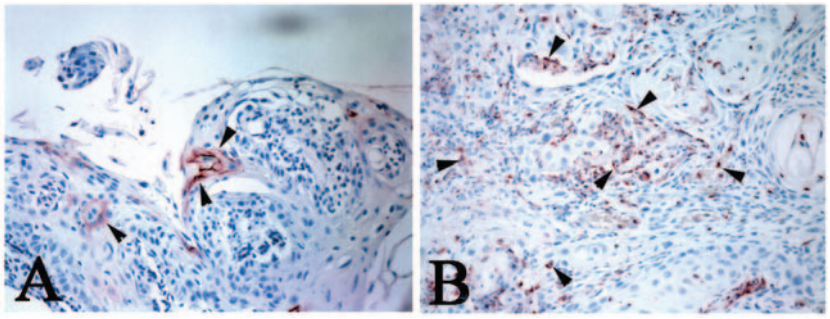

Figure 1. *Detection of pluripotent cells in oral SCC specimens. (A) Biopsy specimens from a well-differentiated oral SCC were incubated in the presence of antibodies to the embryonic stem cell marker TRA-1-60. Well-defined clusters of TRA-1-60 positive cells were found in the specimens. (B) Biopsy specimens from poorly differentiated oral SCC lesions were incubated in the presence of anti-TRA-1-60. In contrast to the clusters seen in the well differentiated lesions the TRA-1-60-positive cells were scattered throughout the specimen.*

described elsewhere (6). SCC9β6 and SCC9β6D1 cells were generated through retroviral transduction with the full–length β6 and β6D1 cDNA (7, 8). The full-length β6 and the construct carrying the β6 C-terminal deletion D1 were generous gifts from Dr Dean Sheppard (UCSF). The β6 deletion D1 lacks the C-terminal 11 amino acids ^{778}EKQKVDLSTDC^{788}. The D1 construct was used to establish the SCC9β6D1 cell line. Neither the SCC9β6 nor the SCC9β6D1 cell lines showed changes to integrins other than β6 (data not shown). The SCC9SN cells, expressing an empty vector, were also established and described elsewhere (7).

Both the SCC9SN and SCC9β6D1 cells have a typical cobblestone appearance and express high levels of E-cadherin indicating a well differentiated epithelial phenotype. In contrast, the SCC9β6 cells are more fibroblast–like in appearance, and do not express much E-cadherin but rather express high levels of the mesenchymal marker vimentin (9). The cells were routinely cultivated in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum at 37˚C with 5% CO_2 and 95% humidity.

Reagents. Rabbit monoclonal antibody 4B5 is directed to the human β6 subunit and was a gift of Dr Robert Pytela (10). Antibodies to TRA160 (MAB 4360) were purchased from Millipore (Chemicon division, Temecula, CA, USA). Monoclonal antibody to CD34 (MEC 14.7) was purchased from Dako (Carpenteria, CA, USA). Actin monoclonal antibodies (SC-10731) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel®, a reconstituted basement membrane was purchased from BD-Biosciences (San Jose, CA, USA).

Wound assay. "Wound" assays were performed by scraping the confluent cell monolayer with a sterile pipette tip. The monolayer was gently washed with PBS to remove cellular debris and then recoated with10 μg/mL FN. At different time points the cultures were stopped, by fixing with 3% paraformaldehyde and processed for immunofluorescence microscopy.

Immunohistochemistry. Multiple 5 μm serial sections from selected formalin-fixed, paraffin-embedded blocks were cut onto aminopropyltriethoxysilane-coated slides. The tissues were dewaxed in xylene and rehydrated. Heat-induced antigen retrieval in citrate buffer (3 min in a pressure cooker) was followed by blockade of endogenous peroxidase activity with hydrogen peroxide. The sections were incubated with 10% goat serum in phosphate-buffered saline to

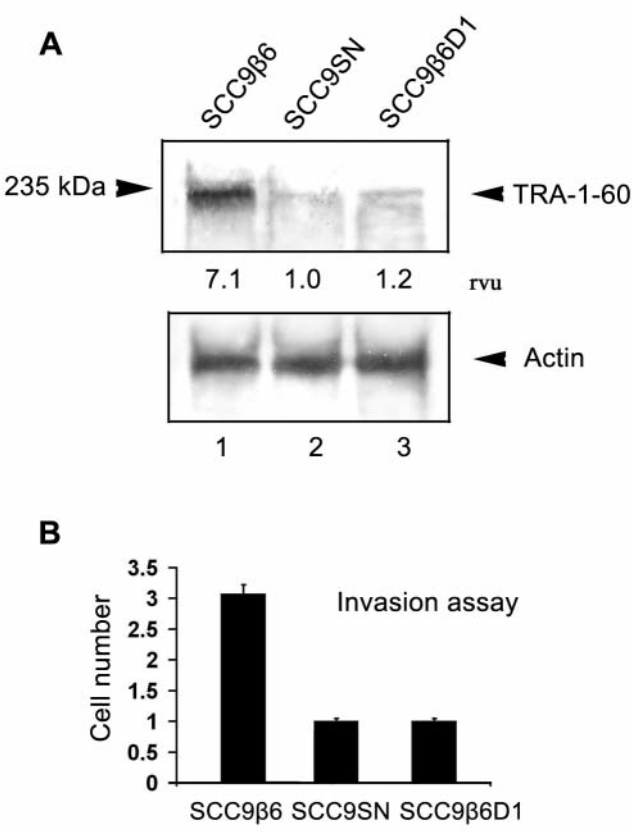

Figure 2. *Differential expression of TRA-1-60 in vitro. (A) 2×10^5 SCC9SN, SCC9β6 and SCC9β6D1 cells were plated onto fibronectin (10 μg/mL) for 24 h. The cells were lysed and the lysate was separated by SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting with anti-TRA-1-60 antibodies. The membrane was quantified by densitometry. The SCC9β6 cell lysate (lane 1) expressed more than 7-fold the level of TRA-1-60 as compared to the lysates from the SCC9SN (lane 2) or the SCC9β6D1 (lane 3) cell lines. (B). 2×10^5 SCC9β6 and SCC9β6D1 cells were seeded serum-free onto Matrigel-coated Transwell filters (8 μm-pore size) and examined for invasion. After 24 h, the filters were fixed, wiped clean and stained with crystal violet. The number of cells crossing the filter was determined. Invasion by the SCC9β6D1 cells was suppressed by 60% compared to the SCC9β6 cells.*

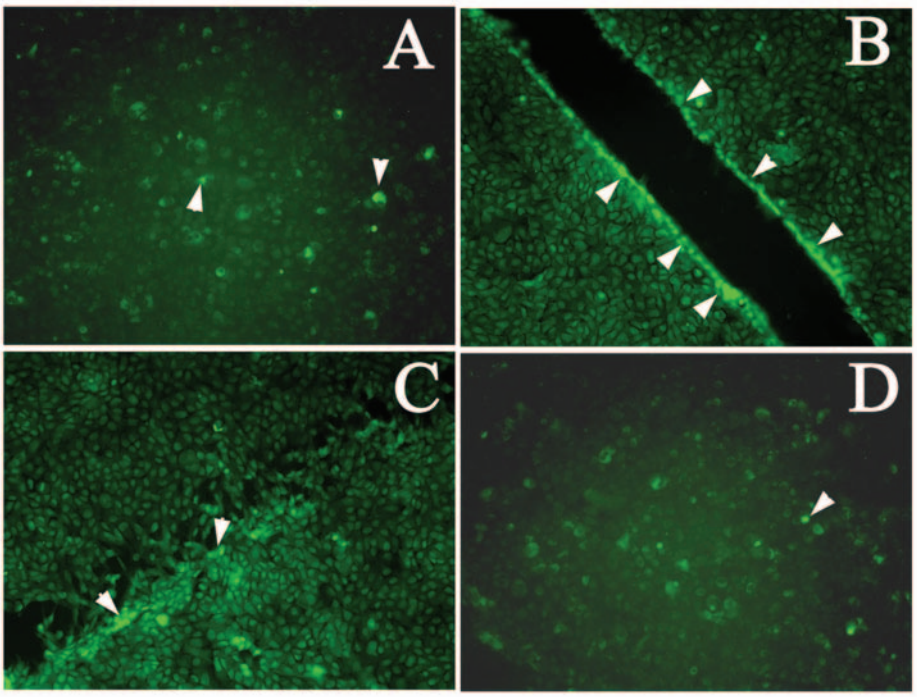

Figure 3. *Pluripotent cells identified at leading edge of a wound. Oral SCC9β6 cells were grown to confluence as a monolayer (A). The monolayer was then wounded with a sterile pipette tip and monitored over time for localization of TRA-1-60. At each time point the culture was stained with antibodies to TRA-1-60. Note the random distribution of TRA-1-60 cells prior to wounding (A). Three hours after wounding the edge of the wound was almost entirely populated by TRA-1-60-positive cells (B). After 24 h when the wound was newly closed, the TRA-1-60 cells were concentrated along a central ridge representing where the two edges of the wound came together (C). After 48 h, the TRA-1-60-positive cells were again randomly distributed throughout the culture (D). These results indicate an important role of the pluripotent cell type in oral cancer cell migration.*

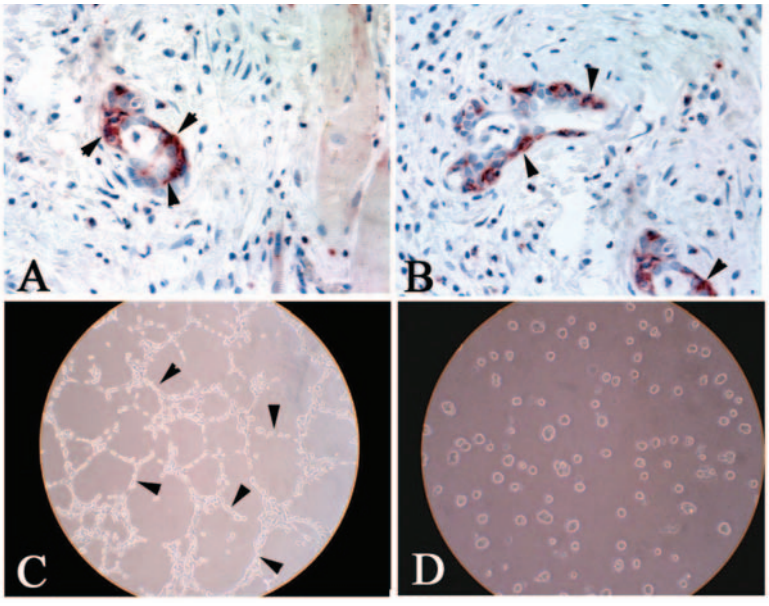

Figure 4. *Vascular Mimicry in oral SCC. In vivo: Biopsy specimens of oral SCC cells containing duct like structures were examined for the expression of TRA-1-60 (A) and αvβ6 (B). Specimens were incubated with a 1:50 dilution of anti-TRA-1-60 (MAB4360) and rabbit monoclonal antibody 4B5 (10) against the integrin β6 subunit (a generous gift of Robert Pytela). The epithelial ducts were positive for both TRA-1-60 and αvβ6, respectively. In vitro: 2×10^5 SCC9β6 (C) and SCC9SN cells (D) were plated onto Matrigel, under serum free conditions. The cells were followed for 24 h. After 24 h the SCC9β6 cells (C) formed a network of interconnecting channels. In contrast the SCC9SN cells remained either as single cells or as clustered groups of spheroids (D).*

reduce non-specific binding and background staining. Anti-TRA-1-60 primary antibody was used at 1:50 dilution (MAB4360) and then applied onto the slides and incubated 1h at room temperature (RT). The slides were washed with Tris-buffered saline containing 0.05% Tween. This was followed by incubation with secondary goat anti-mouse IgM conjugated with horseradish peroxidase (HRP) antibody at room temperature. The color change reaction was performed using diaminobenzidine (DAB) as substrate. The slides were then counterstained with haematoxylin, dehydrated and cleared in xylene. Coverslips were applied with cytoseal 60 mounting medium.

Immunofluorescence microscopy. A total of 2×10^5/mL cells were plated onto fibronectin (FN)-coated glass coverslips (10 μg/mL) for 24 h, serum-free and then fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton® X-100. The cells were incubated first with anti-mAb TRA-1-60 (Chemicon) for 1 h and rinsed with phosphate buffered saline (PBS) and incubated with biotin-conjugated goat anti-mouse IgG (1:50) for 30 min at room temperature followed by an additional rinse with PBS. The cultures were then incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:100) (Amersham, Piscataway, NJ, USA) for 30 min at room temperature, washed with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). The cultures were then examined for expression of green-fluorescence using immunofluorescence microscopy.

Angiogenesis assay. SCC9β6, SCC9β6D1 and SCC9SN cells (2×10^5) were plated on a thin layer of Matrigel® under serum free conditions overnight. As the cells migrate/invade, they establish tunnel or channel like voids within the matrix (11). The cultures were then examined for channel formation.

Western blotting. Cells were seeded at 70% confluence and deprived of serum for 16 hours. The cells were then detached and replated on FN-coated dishes (10 μg/ml) at 37˚C. Cells were collected by scraping and lysed in 150 mM NaCl, 1 Mm EDTA, 20 mM Tris-HCl, 50 mM NaF, 1 mM NaVO4, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.05% aprotinin. The conditioned medium was also collected. Next, the lysates and medium were spun down at 15,000g for 15 min and precleared for 1 h at 4˚C with protein A-agarose. Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were then separated by SDS-PAGE followed by Western blotting using anti-TRA-1-60 antibodies. The immunoblots were visualized by the ECL system and Hyperfilm X-ray film (Amersham).

Cell invasion assay. Cell invasion assays were carried out as described previously (12). Briefly, Transwell filters (Costar, Cambridge, MA, USA) were coated), were coated with Matrigel on the upper surface of the filter for 1 hr at 37˚C to allow polymerization. The lower chamber contained peritumor fibroblasts (PTF) conditioned serum-free medium. Cells were detached with 0.025% trypsin and 0.53 mM EDTA (Invitrogen) and neutralized with trypsin inhibitors (Sigma). Cells were then resuspended in serum-free DMEM. The cells were then placed onto the upper surfaces of the filters and the chambers were incubated at 37˚C for 16 hr. Cells that migrated to the bottom surface of the filter were fixed with methanol, stained with crystal violet, and counted. Each experiment was repeated in triplicate wells, and within each well counting was done in four randomly selected microscopic fields (×200 magnification).

Results

Identification of stem cells in oral SCC. Tissue sections from well differentiated oral SCC were evaluated by immunohistochemistry. The lesions were examined using a mouse monoclonal antibody to the pluripotent cell marker TRA-1-60. Typically, clusters of TRA-1-60–positive cells were identified in some regions of the tumor (Figure 1 A, see arrows). In other areas individual cells stained with TRA-1-60 could also be seen. TRA-1-60 was localized to both the cytoplasm and to the cell membrane (Figure 1A). Results presented are typical of those obtained for 6 random samples.

Differential expression of TRA-1-60 cells in vivo. It was suspected that the relative presence of pluripotent cells in each lesion might reflect the differentiation state of the SCC tumor. Therefore 6 biopsy specimens from poorly differentiated oral SCC were examined by immunohistochemistry (Figure 1B). The lesions were widely decorated with TRA-1-60-positive pluripotent cells suggesting these lesions may be more plastic and hence invasive due to the presence of pluripotent cells (Figure 1B). This was in direct contrast to the isolated clusters of TRA-1-60-positive cells found in the more-differentiated lesions (Figure 1A). It is suggested that the presence of pluripotent cells may reflect and thus impact the aggressive nature of some poorly differentiated oral lesions.

Expression of TRA-1-60 is differentially expressed in oral SCC in vitro. Having identified pluripotent cells in the biopsies, it was necessary to define the relative contribution of stem cells to SCC cell cultures. The highly invasive oral SCC9β6 cells were compared with the poorly invasive oral SCC9SN cell line for the expression of TRA-1-60 (Figure 2 A and B, respectively). It was previously shown that the SCC9SN cells were well differentiated; whereas the SCC9β6 cells were poorly differentiated based on morphology and expression of vimentin and keratin (9). The well differentiated SCC9β6D1 cells, which express a truncated β6 subunit (Figure 2) (9) were also examined. Briefly, the oral SCC cells were plated onto fibronectin (10 μg/mL) for 24 h. The cells were then removed from their substrate, lysed and examined by Western blotting for the expression of TRA-1-60. Using monoclonal antibodies, a proteoglycan was identified in the 230 kDa range corresponding to TRA-1-60. When quantified by densitometry, expression of TRA-1-60 was found to be 7-fold greater in the lysate from the SCC9β6 cells (Figure 2A, lane 1) compared to the lysate from both the SCC9SN and the SCC9β6D1 cell lines (Figure 2A, lanes 2 and 3; respectively). These results indicate that the β6 integrin is an important regulatory component of differentiation and

its full-length expression is necessary for maintenance of the undifferentiated state as demonstrated for by the expression of TRA-1-60.

Deletion of the β6 C-terminal 11 AA suppresses invasion. The SCC9β6D1 cells were compared to the SCC9β6 cell line for invasion of a reconstituted basement membrane (Matrigel). Briefly, 2×10^5 SCC9β6 and SCC9β6D1 cells were seeded onto Matrigel-coated 8 μm-pore size filters under serum-free conditions. After 24 h the upper surface of the filter was wiped clean, fixed and stained with crystal violet. The number of invading cells was determined visually. Three-fold as many SCC9β6 cells crossed the Matrigel barrier when compared to the SCC9SN and the SCC9β6D1 cell line. The results indicate that the SCC9β6 cells expressing the full length β6 subunit are significantly more invasive than cells expressing no β6 or those cells expressing a truncated β6 integrin subunit. Clearly the β6 cytoplasmic domain is an important regulatory component of invasion and pluripotency as expression of TRA-1-60 is also dependent upon expression of the full length β6 subunit.

Closure of an experimental wound is modulated by TRA-1-60 positive cells. A wound assay was used to examine the contribution of TRA-1-60 cells to migration. Briefly, the SCC9β6 cells were grown to confluence as a monolayer on a fibronectin substrate and then "wounded" using a sterile pipette tip (Figure 3). The cultures were then followed for closure of the gap over a 24 h period. At various time points the cultures were fixed, permeabilized and stained with anti-TRA-1-60 monoclonal antibodies, followed by anti-mouse FITC and processed for immunofluorescence microscopy.

The results were as follows: Prior to wounding, the distribution of TRA-1-60 cells was randomly scattered throughout the monolayer (Figure 3 A). At 3 h after wounding, and prior to cell movement, the entire edge of the wound consisted primarily of TRA-1-60-positive cells (Figure 3 B; arrows). As the cells moved into the wound, the leading edge was consistently populated by TRA-1-60-positive cells; indicating that pluripotent cells play an active role in the initiation and the subsequent closure of the "wound". These results demonstrate that as a monolayer of oral SCC cells migrates or invades, that pluripotent cells align themselves at the leading edge of the wound. When the wound is newly closed, there is a residual "seam" of pluripotent cells (+TRA-1-60) indicating the site where the two edges came together (Figure 3C). Once the monolayer is re-established the TRA-1-60 cells disperse back into the general population (Figure 3D). These results indicate that pluripotent cells are at the leading edge of a closing wound and may be prominent contributors to the invasion of oral SCC.

Vascular channel formation in oral SCC. Human biopsy specimens: During routine examination of several oral SCC specimens, epithelial cells clustered into "duct-like" structures were identified. All of these ducts were positive for both TRA-1-60 and the epithelial specific–integrin, αvβ6 (Figure 4 A and B; respectively). The expression of TRA-1-60 indicates a pluripotent status and the expression of αvβ6 indicates that the cells are epithelial tumor cells. Leukocytes and erythrocytes detected in the lumen of these structures suggested the presence of vascular mimicry (13). Vascular mimicry is a form of angiogenesis in which the tumor cells form vascular channels to compensate for the lack of blood vessel production in order to provide nutrients for the tumor (13). The tissue sections were negative when stained with antibodies to the endothelial cell marker CD34, further indicating these blood vessels were not composed of endothelial cells but rather composed of oral SCC cells. These results are consistent with respect to the work of Dr Mary Hendrix (personal communication). However, this is the first identification of vascular mimicry associated with oral cancer.

Vascular channel formation by SCCβ6 cells in vitro. Can vascular mimicry be analyzed *in vitro*? The controversial idea that tumor cells can form auxiliary blood vessels was originally postulated by Dr Hendrix (13). However, this has never been reported for oral SCC. Based on preliminary experiments, examination of whether this could be evaluated *in vitro* was performed. Channel formation between the highly invasive SCC9β6 cells and the poorly invasive SCC9SN and SCC9β6D1 cells was compared.

A total of 2×10^5 cells were seeded onto a thin layer of Matrigel and followed for subsequent channel formation. After 24 h, under serum free conditions, the SCCβ6 cells formed a complex network of interconnecting channels (Figure 4C) whereas the SCC9SN cells did not (Fig 4D). In contrast, the SCC9SN cells remained as single cells or in some instances formed small spheroids of clustered cells (Figure 4D). The SCC9β6D1 cells (Figure 4E) also remained as single cells or as spheroids much like the SCC9SN cells. These results suggest that the ability to invade and form vascular channels is dependent upon the full length β6 subunit and that truncation of the 11 AA from its C-terminus suppresses this behavior. This suggests that vascular mimicry is mediated by signals contained within the full length β6 sequence.

Discussion

Invasive oral SCC is a disease in which the tumor cells exhibit a significant degree of plasticity. This plasticity is part of normal disease progression and is reflected during the progression of oral SCC through classical epithelial-

mesenchymal transition (EMT). During progression through EMT, specific markers of the mesenchymal phenotype are upregulated and some markers associated with epithelial phenotype are decreased (9). It has been suggested that transformation of a normal cell into a cancer cell requires 3 to 5 distinct events (14). Therefore, only long-term residents of the epidermis, presumably stem cells, would have the ability to acquire the required number of genetic hits necessary (14). During this process SCC cells progress from having an epithelial profile to a more mesenchymal profile in both morphology and behavior (9). It was recently demonstrated that the expression of the β6 integrin is crucial to maintenance of the mesenchymal phenotype (9).

In addition to nucleic acids, proteins and lipids, oligosaccharides and polysaccharides (glycans) are the fourth major class of cellular macromolecules (15). Glycans are often attached to proteins and lipids to form a dense glycocalyx on the surface of all cells, including embryonic and pluripotent stem cells (15). The glycocalyx is optimally positioned to help the stem cell communicate with its niche (15). The tumor-rejection antigens (TRA) are a family of widely used markers of embryonic stem cells. TRA-160 has been shown to recognize a keratan-sulfated proteoglycan (KSPG) and is highly expressed in embryonic stem cells. Upon differentiation TRA-1-60 vanishes (15).

Monoclonal antibodies to TRA-1-60 were used to evaluate the contribution of pluripotent cells to oral cancer progression.

Discrete clusters of SCC cells which were positive for the pluripotent cell marker TRA-1-60 were successfully identified. The presence of the putative stem cells in oral cancer was not surprising as it has been previously shown that stem-cells are a component of oral SCC (2). It was particularly interesting to see if there was a correlative difference between the number of stem cells in well differentiated and poorly differentiated lesions. It was found that TRA-1-60 expressing cells were scattered throughout the undifferentiated lesions, which was in direct contrast to the localized cell clusters demonstrated in the well differentiated lesions.

Adult or somatic stem cells generally have limited function without their niche. A case in point is the hematopoietic stem cells which regenerate the entire blood and immune system and makes copies of itself after limiting-dilution transplantation. Hematopoietic stem cells circulate freely but have little function outside specific anatomic sites (16). It is the specific cues from specific sites that allow stem cells to persist and to change in number and fate. Some stem cells might have a niche composed of extracellular matrix and other non-cellular constituents that could regulate their control (14).

In normal oral mucosa the constant renewal process is ultimately dependent on somatic stem cells, a small subset of cells with phenotypically and behaviorally distinct properties. These cells are endowed with a seemingly unlimited capacity for self-renewal and the ability to generate cells that differentiate to maintain tissue structure and function (17). Based on these facts the pluripotent cell population was tested within a 3 dimensional matrix composed of fibronectin as well as the laminin rich EHS-matrix (Matrigel®).

A subpopulation of cells was identified within the lesion that appear to have the plasticity of stem cells as observed using antibodies to TRA-160, a stem cell marker (Figure 1). Most cells in cancer have a limited ability to divide but only a small subset of phenotypically distinct cells have the capacity to self renew and form new tumors (5).

The ability to nourish a tumor is an important rate-limiting aspect of tumor growth and the influx of new blood vessels is pivotal to this process. The ability of invasive oral cancer cells to manifest endothelial-like qualities allows them to adapt to the hypoxic microenvironment associated with rapidly growing tumors and at the same time enables them to circumvent conventional anti-angiogenic therapies which are directed to endothelial cell specific revascularization.

Duct like structures were identified in the tumors appearing to be a form of vascular mimicry (13). These ducts which were TRA-1-60 and αvβ6-positive were localized for the first time in human oral cancer biopsy specimens (Figure 3). When cells in culture were examined, the SCC9SN cells did not form networks but rather formed extensive cell-cell clusters or remained as single cells. In contrast, when the SCC9β6 cells were used for the assay, they formed an extensive network of channels. It was surprising to observe that the SCC9β6D1 cells remained as single cell isolates or formed clusters (spheroids). This clearly demonstrates a most important role for the cytoplasmic domain of the integrin β6 in promoting the formation of "vascular" networks. These results suggest that perhaps much of the regulatory information on vascular mimicry may be modulated by the cytoplasmic tail of the β6 integrin. The results of this study clearly add to the ever-growing body of literature with respect to αvβ6 and oral cancer. The data clearly suggests that within the oral SCC lesion there is a subpopulation of cells that are pluripotential. These cells appear to be important in allowing the tumor to have a ready line of replacement cells and these cells can for all practical purposes be converted to aggressive tumor cells or in many cases contribute to tumor growth *via* vascular mimicry. Most interesting and novel is the finding that the αvβ6 is an important regulator of pluripotential cell behavior but also of vascular mimicry. Perhaps this difference in distribution of pluripotent cells is ultimately reflective of cell behavior and invasive potential.

Acknowledgements

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