Published in final edited form as:

Exp Cell Res. 2013 April 15; 319(7): 1028–1042. doi:10.1016/j.yexcr.2013.01.013.

Inherent phenotypic plasticity facilitates progression of head and neck cancer: endotheliod characteristics enable angiogenesis and invasion

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Abstract

The presence of the EMT (epithelial-mesenchymal transition), EndMT (endothelial-mesenchymal transition) and VM (vasculogenic mimicry) demonstrates the multidirectional extent of phenotypic plasticity in cancers. Previous findings demonstrating the crosstalk between head and neck squamous cell carcinoma (HNSCC) and vascular endothelial growth factor (VEGF) imply that HNSCC cells share some functional commonalities with endothelial cells. Our current results reveal that cultured HNSCC cells not only possess endothelial-specific markers, but also display endotheliod functional features including low density lipoprotein uptake, formation of tube-like structures on Matrigel and growth state responsiveness to VEGF and endostatin. HNSCC cell subpopulations are also highly responsive to transforming growth factor- β 1 and express its auxiliary receptor, endoglin. Furthermore, the endotheliod characteristics observed *in vitro* recapitulate phenotypic features observed in human HNSCC tumors. Conversely, cultured normal human oral keratinocytes and intact or ulcerated human oral epithelia do not express comparable endotheliod characteristics, which imply that assumption of endotheliod features is restricted to transformed keratinocytes. In addition, this phenotypic state reciprocity facilitates HNSCC progression by increasing production of factors that are concurrently pro-proliferative and pro-angiogenic, conserving cell energy stores by LDL internalization and enhancing cell mobility. Finally, recognition of this endotheliod phenotypic transition provides a solid rationale to evaluate the antitumorogenic potential of therapeutic agents formerly regarded as exclusively angiostatic in scope.

Keywords

Phenotypic state reciprocity; Endotheliod characteristics; Transforming growth factor (TGF); endoglin; Head and neck squamous cell carcinoma (HNSCC)

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common human cancer worldwide and the eighth leading cause of cancer death with a five year

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survival rate as low as 50% [1, 2]. Substantial research has focused on identification of the underlying molecular and biochemical mechanisms associated with HNSCC tumorigenesis in order to facilitate development of disease-specific treatments [3–5]. Such mechanistic insights have enabled development of more targeted chemotherapeutic agents, including a spectrum of growth factor specific antagonists, e.g. cetuximab and bevacizumab, to suppress EGF and VEGF signaling, respectively [6, 7]. Previous investigations, by our lab and others, demonstrated that the angiostatic agent, endostatin, inhibited migration and invasion of HNSCC cells, implying that HNSCC cells share some functional characteristics with endothelial cells [8, 9]. In subsequent studies, we demonstrated that HNSCC cells produce exceptionally high levels of VEGF, express both VEGFR1 and VEGFR2, and proliferate in response to autologously produced VEGF [10]. This autocrine-paracrine VEGF growth loop can promote HNSCC tumorigenesis in a biphasic fashion via its proangiogenic and growth promotion roles [10]. Importantly, these VEGF-HNSCC *in vitro* interactions recapitulate components of the premalignant lesion transformation to overt cancer including the increased vascular density that accompanies development and subsequent progression of HNSCC [10, 11]. Collectively, these findings suggest an inherent phenotypic plasticity toward endothelial features in a subset, if not the entire population, of HNSCC cells.

The presence of variable phenotypic modulations, including epithelial to mesenchymal transition (EMT), mesenchymal to epithelial transition (MET), endothelial-mesenchymal transition (EndMT) and vasculogenic mimicry (VM), indicate a vast extent of multidirectional phenotypic plasticity among tumor cells and their corresponding stroma [12–16]. Most of these transitions provide tumor-promoting effects such as assumption of an invasive phenotype during the EMT [12, 17], release of the tumor promoting factors TGF- β and VEGF by cancer associated fibroblasts which originate from transformed endothelial cells that have undergone the EndMT [14, 18], and acquisition of *de novo* microcirculation and blood supply by forming functional vascular-like channels during cancer cell VM [15, 19, 20]. While VM describes one potential aspect of the functional significances underlying the assumption of endothelial characteristics by malignant tumor cells [19–21], based on our previous findings regarding the HNSCC-VEGF crosstalk [10] and additional basic and clinical research papers [6, 8, 9], we hypothesized that the endothelial features of HNSCC cells extend beyond formation of vessel-like VM networks.

The purpose of this study was to explore and compare the extent of endothelial functional characteristics present in both *in vitro* cultured HNSCC cell lines as well as primary and metastatic HNSCC tumors relative to corresponding normal oral keratinocytes and oral mucosa. Our results demonstrate that the extent of phenotypic plasticity is reduced and much more transient in normal keratinocytes relative to fully transformed HNSCC cells and tissues. These data also imply that the epithelial-endothelial transition is highly supportive for tumorigenesis by virtue of the corresponding increased production of growth factors that are both pro-proliferative and angiogenic, assumption of a more mobile phenotype, and conservation of cellular energy stores. Recognition of this unique phenotypic state reciprocity encourages evaluation of the antitumorigenic potential of therapeutic agents formally regarded as exclusively angiostatic in scope.

Materials and Methods

Due to the acknowledged inter- and intra-HNSCC tumor heterogeneity, these studies employed three well characterized HNSCC cell lines and evaluated 24 HNSCC tumor samples.

Cell Culture

Three HNSCC cell lines (CAL27/CRL-2095, SCC9/CRL-1629, and SCC15/CRL-1623), derived from human tongue cancers were obtained from the American Type Cell Culture (ATCC, Manassas, VA). For expansion, cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) at 37°C, 5% CO₂. In order to avoid the interfering effects from serum components, cells were cultured in serum free medium for selected experiments. Human oral keratinocytes (HOK) were purchased from ScienCell (Carlsbad, CA) and cultured in oral keratinocyte medium (OKM +OKGS+PIS, ScienCell). U937 monocytes (ATCC) were incubated in RPMI-1640 medium supplemented with 10% FBS (GIBCO, Grand Island, NY) at 37°C, 5% CO₂. Human umbilical vein endothelial cells (HUVECs, ScienCell) were grown in endothelial cell medium (ECM) supplemented with 5% FBS, 1% endothelial cell growth supplement (ECGS) and 1% penicillin/streptomycin (P/S) at 37°C, 5% CO₂ (ECM, ECGS and antibiotics were obtained from ScienCell). Transforming growth factor beta 1 (TGF-β1) is a recognized acute phase reactant associated with cell phenotypic changes, e.g., EMT. For selected experiments, cells were exposed to recombinant human TGF-β1 (BioLegend, San Diego, CA) to assess its effects on the endothelial phenotypic transition.

Immunocytochemistry

HUVEC, HOK and HNSCC cells (1×10⁴ cells/chamber) were seeded on chamber slides followed by 24 h incubation in serum free base medium with or without 10 ng/ml recombinant human TGF-β1 (BioLegend) at 37°C, 5% CO₂. Following routine wash, fixation steps, cell monolayers were then blocked with 1% BSA for 30 min and incubated with VE-Cadherin (1:50, Santa Cruz, Biotechnology, Santa Cruz, CA), CD31 (1:100, Cell Signaling Tec., Boston, MA), vimentin (1:200, Abcam, Cambridge, MA) or cytokeratin 6 (1:200, Santa Cruz Biotechnology) antibodies at 4°C overnight. Cells were then incubated with FITC or Texas Red conjugated secondary antibodies (Abcam, Cambridge, MA) for 1 h at room temperature. DAPI was used to visualize the nuclei. Fluorescence microscopy images were obtained by using an Olympus BX51 microscope (Olympus, Japan), Nikon DS-Fi1 digital camera (Nikon, Japan) and ImagePro 6.0 software (MediaCybernetics, Bethesda, MD).

Western Blot analysis

HNSCC, HUVEC or HOK cells were harvested by using Accutase™ cell detachment reagent (Chemicon, Temecula, CA). Total cytoplasmic and nuclear protein was extracted from the cell pellets by M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). Western blot analyses were then conducted using the previously described method [10]. The antibodies and working dilutions were as follows: Erk1/2 mouse monoclonal antibody (1:2000, Cell Signaling Tec.), and phospho-Erk1/2 rabbit polyclonal antibody (1:1000, Cell Signaling Tec.), Src rabbit monoclonal antibody (1:1000 dilution, Cell Signaling Tec.), phospho-Src rabbit polyclonal antibody (1:1000 dilution, Cell Signaling Tec.), VE-cadherin mouse monoclonal antibody (1:200, Santa Cruz Biotechnology). Kodak 1D3 image analysis software (Kodak) was employed to perform densitometric analyses. Data were normalized relative to protein levels of β-actin, which was probed by a mouse monoclonal antibody (1:1000, Santa Cruz Biotechnology).

Qualitative and quantitative assessment of acetylated low density lipoprotein (AcLDL) internalization

Log-growth HNSCC cells, HOKs and HUVECs were seeded on chamber slides (qualitative assay) or 96-well plates (quantitative assay) and pretreated with or without 10 ng/ml recombinant human TGF-β1 (BioLegend) in serum free base medium for 24 h. Conditioned

media were then discarded and cells were exposed to 10 µg/ml Alexa Fluor 488 conjugated AcLDL (Molecular Probes, Eugene, OR) for 4 h in 10% FBS supplemented growth media at 37°C, 5%CO₂. Extracellular Alexa Fluor 488 signals were quenched with 0.08% Trypan Blue (Sigma-Aldrich, St Louis, MO), followed by two washes with phosphate buffered saline (PBS). Intracellular Alexa Fluor 488-AcLDL uptake was qualitatively observed using an Olympus BX51 fluorescence microscope. In the qualitative assay, E-Cadherin (mouse monoclonal antibody, 1:50, Abcam Inc) and CD31 (1:100, Cell Signaling Tec.) staining were used to delineate HNSCC, HOKs and HUVECs intercellular boundaries, respectively. DAPI was used to visualize the nuclei. Cellular fluorescent images were acquired using a Nikon DS-Fi1 digital camera and the ImagePro 6.0 software. The FLUOstar Omega microplate reader (BMG Labtech, Germany) was employed to quantify cellular AcLDL uptake. An AcLDL standard curve was conducted with each assay. U937 monocytes were also included in the quantitative assay as a positive control. Quantitative data were normalized to AcLDL (ng) per 10⁵ cells. Cell numbers were determined by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) incorporating cell line-specific standard curves.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Twenty-four hour serum deprived HUVECs and HNSCC cell cultures were incubated with or without 10 ng/ml recombinant human TGF-β1 (BioLegend, San Diego, CA) for 48 h in serum free base medium. Conditioned media were collected and concentrated with Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, MA). Total cellular proteins were then extracted from the cell monolayers by using M-PER. Protein levels of Endoglin present in both conditioned media and total cell lysates were analyzed by protein specific DuoSet ELISA Development Kits (R&D Systems, Minneapolis, MN). Concentrations of target proteins were normalized to pg/mg total protein.

Cell Invasion Assay

Following 24 h serum deprivation, conditioned media were removed and HNSCC cells were pretreated with 0 (negative control) or 10 ng/ml recombinant human TGF-β1 (BioLegend) in fresh, serum free, base medium for an additional 24 h prior to the cell invasion assay (InnoCyte cell invasion kit, Calbiochem, San Diego, CA). Cells (3.5 × 10⁵/well) were then seeded in the upper chambers with or without 10 ng/ml TGF-β1 in fresh, serum free, base medium. Media supplemented with 10% FBS were added to the lower chambers as chemoattractants. The invasion plates were then incubated at 37°C, 5% CO₂ for 48 h with fresh TGF-β1 (10 ng/ml) added every 24 h. Standard curves consisting of cell line matched control cells were run concurrently with each invasion assay.

Immunohistochemistry

Twenty-four primary or metastatic human HNSCC tumor samples were obtained from the Ohio State University Comprehensive Cancer Center Tissue Procurement Services (in accordance with Ohio State University IRB approval) and were immediately placed in 10% neutral buffered formalin (24–48 h). The histopathology reports containing information of patients' gender, age, clinical sites, etc. were provided by the Tissue Procurement Services. Five histologically normal and ten ulcerated nonneoplastic oral mucosal tissues were randomly chosen from achieved oral pathology biopsy samples (IRB approved). Paraffin embedded tissue sections were deparaffinized, rehydrated and microwaved in citrate buffer (pH 6.0) for antigen retrieval. Sections were then blocked with 5% normal serum, 1% bovine serum albumin (Sigma-Aldrich), 0.05% Tween[®] 20 (Fisher Scientific, Pittsburgh, PA) in PBS for 1 h and incubated with primary antibodies or PBS (negative control) at 4°C overnight, followed by incubation with biotinylated secondary antibodies and Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). Antibodies employed in this study

were: VE-cadherin (1:10, Millipore), CD31 (1:100, Cell Signaling Tec.), vimentin (1:100, Abcam), Pan-cytokeratin (1:75, Abcam).

Three dimensional cell culture and tube formation assay

Log growth HUVEC, HOK and HNSCC cells were trypsonized and resuspended to 4×10^5 cells/ml with 10% FBS supplemented growth media. Cell suspensions (300 μ l/well) were then added to Matrigel (289 μ l/well, BD Biosciences, Bedford, MA) pre-coated 24-well plates and incubated at 37°C, 5% CO₂ for 17 hrs. Formation of tube-like structures was observed using a Zeiss Axiovert 200 phase contract inverted microscope (Zeiss, Germany). Digital images were acquired using a Carl Zeiss AxioCam MRC5 digital camera (Zeiss, Germany).

Statistical analyses

TGF- β 1's effects on AcLDL uptake and endoglin protein expression levels in individual HNSCC cell lines were evaluated using the Mann Whitney U two-tailed test. Results of cell invasion assay were analyzed using the Wilcoxon one-tailed signed rank test. Comparison of inter-cell line AcLDL uptake was conducted using the Kruskal-Wallis Analysis of Variance, followed by a Dunn's Multiple Comparisons post-test. The data distribution determined whether parametric or non-parametric analyses were conducted.

Results

Cultured HNSCC cells express endothelial-associated proteins VE-cadherin, CD31, and Vimentin

Consistent with their endothelial lineage, cultured HUVECs showed the presence of three "endothelial markers": 1) the vascular endothelial specific intercellular adhesion molecule VE-cadherin (Figure 1A), 2) the platelet endothelial cell adhesion molecule PECAM-1 (CD31, see Figure 2B and Figure 3A) and 3) vimentin, the intermediate filament which is typically found in mesenchymal and endothelial cells (Figure 2A). Corresponding immunocytochemistry (ICC) analyses of HNSCC cells confirmed the presence of VE-cadherin (Figure 1A), vimentin, and CD31 in all three HNSCC cell lines evaluated (Figure 2 C–H). The presence of VE-cadherin protein was also verified by immunoblotting in all three HNSCC cell lines (Figure 1B). Furthermore, the extent of expression was both marker and HNSCC cell line dependent, with highest VE-cadherin levels detected in SCC15 cells (Figure 1B). HOKs, in contrast, did not express VE-cadherin (Figure 1A). The epithelial origin of HNSCC cells and HOKs was confirmed by E-cadherin staining shown on Figure 4A and Figure 5A.

HNSCC cells form tube-like structures on Matrigel 3D cultures

The capacity to form tube-like structures on Matrigel is a recognized feature of vasculogenic mimicry [15, 23]. Similar to HUVECs, all 3 HNSCC cell lines demonstrated formation of tube-like structures following 17h three-dimensional culture on Matrigel (Figure 3). Notably, the tube-like structures formed in HUVEC cultures were lined by a single layer of HUVEC cells. Their counterparts in HNSCC cultures, however, were surrounded with irregularly arranged HNSCC cell aggregates (Figure 3). The tube-like structure morphology was dynamic and the tubules themselves were obliterated within 3 days due to expansion of cell population. HOKs failed to show the similar capacity of tube formation following 3D culture on Matrigel (Figure 3).

HNSCC cells internalize AcLDL, which is augmented following exposure to TGF- β 1

As AcLDL uptake is an established characteristic of endothelial cells and macrophages [24–26], HUVECs and U937 monocytes served as the respective control cell populations for these studies. Both qualitative and quantitative analyses confirmed the ability of HNSCC cell lines to internalize AcLDL. While qualitative AcLDL levels were highest in HUVECs (Figure 4A.), all three HNSCC cell lines evaluated (CAL27, SCC9 and SCC15) demonstrated cytoplasmic internalization of Alexa Fluor 488 conjugated AcLDL (Figure 4A). Normal HOKs, however, failed to show any evident AcLDL uptake (Figure 4A). Due to their lack of adherent growth, U937 cells were excluded from these qualitative studies. Quantitative analyses showed that among the HNSCC cells, CAL27 cells exhibited the greatest internalization of Alexa Fluor 488-AcLDL, which was comparable to the control HUVECs population ($46.7 \pm 2.3\%$, $P > 0.05$, $n = 8$) (Figure 4B). Internalized AcLDL levels achieved in SCC9 and SCC15 cells were significantly lower than control HUVECs [$33.5 \pm 0.8\%$, $P < 0.01$, $n = 8$, SCC9) and ($16.8 \pm 1.0\%$, $P < 0.001$, $n = 8$, SCC15)] (Figure 4B). Finally, 24 h treatment of HNSCC cells with 10 ng/ml of the known phenotypic modulating agent, TGF- β 1, significantly increased cellular capacity to internalize AcLDL in all three HNSCC cell lines (Figure 4B). While TGF- β 1 treated SCC15 cells demonstrated a $29.4 \pm 9.0\%$ ($P = 0.0207$, $n = 8$) increase of AcLDL uptake relative to the non-treated SCC15 cells, the total internalized AcLDL levels in treated SCC15 cells were still significantly lower relative to HUVECs ($P < 0.001$, $n = 8$) and treated CAL27 cells ($P < 0.01$, $n = 8$). Although AcLDL uptake is known to be mediated by scavenger receptors in macrophages and endothelial cells [27, 28], we did not identify the presence of the scavenger receptors (SR-A, CD36) in cultured HNSCC cells in this study (data not shown). These findings imply HNSCC internalization of AcLDL occurred by another mechanism.

Introduction of TGF- β 1 redirects expression of HNSCC cell adhesion molecules towards a less adherent, more mobile “endotheliod” phenotype

A 24 h exposure of SCC15 cells to 10 ng/ml TGF- β 1 elicited striking modulations in cell adhesion molecules. Notably, TGF- β 1 treated SCC15 cells demonstrated “cadherin switching” as manifested by a marked decrease in E-cadherin with a concurrent increase in VE-cadherin. In addition, levels of CD31 and vimentin were also increased in treated SCC15 cells (Figure 5A). Interestingly, CAL27 and SCC9 cultures’ cell adhesion molecules were refractory to TGF- β 1 challenge (data not shown). Repeated experiments demonstrated comparable findings.

TGF- β 1 treatment increases levels of a component of the TGF- β 1 receptor complex, endoglin, in HNSCC cells

The three HNSCC cell lines demonstrated very low constitutive levels of endoglin in cell lysates, which corresponded to $0.81 \pm 0.09\%$ (CAL27), $0.22 \pm 0.14\%$ (SCC9) and $0.42 \pm 0.18\%$ (SCC15) of the endoglin level in positive control HUVECs. Forty-eight hour treatment (10ng/ml TGF- β 1) significantly increased endoglin levels in CAL27 and SCC15 cells by $210.0 \pm 55.6\%$ ($P = 0.0286$, $n = 4$) and $575.3 \pm 85.2\%$ ($P = 0.0286$, $n = 4$), respectively (Figure 5B). SCC9 cells, which were essentially refractory to TGF- β 1 challenge, showed an insignificant decrease ($-34.2 \pm 57.2\%$, $P = 0.8834$, $n = 4$) of endoglin compared to their matched control cells (Figure 5B). Similar to others’ findings which demonstrated that endoglin could be shed and released into stroma as a soluble form [29, 30], extracellular endoglin levels were detected in the conditioned media of cultured HUVECs (380.0 ± 75.3 pg/ml, $n = 2$) and only in one SCC cell line-SCC15 (16.2 ± 5.3 pg/ml, $n = 2$). TGF- β 1 treatment increased endoglin levels in conditioned medium of SCC15 cells to 29.3 ± 1.2 pg/ml ($n = 2$).

TGF- β 1 treatment augments invasiveness of HNSCC cells

We have previously confirmed that all three HNSCC cell lines readily invade synthetic basement membranes in response to chemoattractants [10]. For these current studies, we employed the most TGF- β 1 responsive (SCC15) and TGF- β 1 refractory (SCC9) cell lines. Our data show TGF- β 1 (10 ng/ml, 24 h) treatment significantly increased SCC15 invasion by $36.3 \pm 17.5\%$ ($P = 0.0313$, $n = 5$) (Figure 5C). Similar to the endoglin experiments, SCC9 cells were unaffected by treatment, resulting in an insignificant decrease ($-11.1 \pm 2.6\%$, $P = 0.2188$, $n = 5$) in invasion following TGF- β 1 challenge (Figure 5C).

Surface epithelial cells from biopsies of ulcerated, otherwise normal oral mucosa display enhanced levels of the “mesenchymal” microfilament, vimentin, which is not expressed in intact, normal oral epithelium

Five histologically normal oral mucosal samples with intact epithelium and ten ulcerated, non-neoplastic oral biopsies were evaluated for the presence of VE-cadherin, vimentin, and CD31. Intact oral epithelium was negative for all of these markers. Focal positivity was observed for CD31 and vimentin in the resident antigen presenting cells (Langerhans cells) interspersed among the surface epithelial cells (Figure 6, Column 1). In contrast, biopsies of ulcerated mucosa demonstrated the presence of vimentin in surface epithelial cells, with greater intensity of staining (as red arrows indicate) observed in the basal 1/3 of epithelium adjacent to the ulcerated sites (Figure 6, Column 2). CD31 (Figure 6, Column 2 Row 4) and VE-cadherin (Figure 6, Column 2 Row 5, as the red rectangle indicates) were shown on stromal vascular endothelial cells which served as an internal control. These two endothelial markers, however, were not detected in the ulcerated epithelial cells.

VE-cadherin, Vimentin, and CD31 proteins are also present in lesional cells of primary and metastatic HNSCC tumors

Twenty-four HNSCC tumors, which corresponded to HNSCC clinical stages II through IV, were evaluated for the presence of pan-cytokeratin, VE-cadherin, vimentin, and CD31 by immunohistochemistry (See Figure 6 and Table I). Positive pan-cytokeratin staining confirmed the epithelial origin of the tumor nests (Figure 6, Row 1). Endothelial and connective tissue stromal cells served as the internal controls for VE-cadherin, CD31 (endothelial cells) and vimentin (both endothelial and stromal cells) staining (Figure 6). Tumor nests that approximated regions of tumor necrosis demonstrated the highest level of CD31 and vimentin (Figure 6). VE-cadherin was most prevalent in the regions that contained advancing tumor islands (Figure 6, Column 4 Row 5, as the red rectangle indicates). Depiction of the distribution (as indicated by percent positive staining) of these three markers relative to clinical stage and nodal involvement were summarized in Table II. These data demonstrate that higher percentages of VE-cadherin and vimentin were detected in lesional cells of HNSCC tumors obtained from patients with lymph node metastasis ($N > 0$, i.e., presence of 1 or more metastatic nodes) and more advanced clinical stages ($> II$, i.e., primary HNSCC tumor $> 2\text{cm}$). While these data imply a positive correlation between higher clinical stage and extent of endothelial features, due to the small number ($n = 3$) of lower stage i.e. $< \text{Stage II}$ tumors, correlative analyses were not possible. Relevant to the VM, a number of tube-like structures with lumen exclusively lined with cancer cells were identified in many HNSCC specimens. No blood cells, however, were observed within those structures (data not shown).

VEGF elicits phosphorylation of downstream signaling mediators Erk1/2 and Src in HNSCC cells

We have previously demonstrated that HNSCC cells possess VEGFR1 and VEGFR2, which are receptors that are traditionally regarded as endothelial specific [10]. In this current study,

Western blot results demonstrated time-dependent modulation of phospho-Erk1/2 and phospho-Src in CAL27 cells following 50 ng/ml VEGF challenge (see Figure 7A). Phosphorylation of Erk1/2 and Src occurred rapidly (within 1 min) after addition of VEGF. The phosphorylated levels remained elevated for approximately 20 min and gradually decreased to the basal levels over the subsequent 40 min. As the Src and Phospho-Src antibodies react with all kinase members in Src family [31], multiple bands were observed in Src and Phospho-Src blots (Figure 7A).

Endostatin attenuates VEGF-induced downstream signaling in HNSCC cells

While endostatin is a recognized angiostatic agent and is presumed to primarily target endothelial cells, previous studies by our lab and others, demonstrated that endostatin also inhibited migration and invasion of HNSCC cells [8, 9]. Our current data showed that pretreatment with endostatin (10 μ g/ml) prior to VEGF challenge (50ng/ml, 2 min) attenuated VEGF-induced phosphorylation of Erk1/2 and Src in a time-dependent fashion in CAL27 cells (Figure 7B). Sixty-minute endostatin pretreatment markedly diminished the phosphorylation of Erk1/2 and Src to the baseline levels (Figure 7B).

Discussion

Expression of cell adhesion molecules and the presence of specific intermediate filaments have traditionally been regarded as cell-lineage specific, e.g., cytokeratins-epithelial cells, vimentin-endothelial or mesenchymal origins [32, 33]. Consistent with this concept, identification of these “lineage specific” molecules by techniques such as IHC is currently a standard diagnostic tool used by pathologists to aid in the diagnosis of lesions of uncertain histogenesis. The recognition of the EMT, MET, EndMT and VM, however, transformed this traditional cell-lineage specific paradigm [12–15]. Our data, which demonstrate the presence of molecules typically associated with endothelial cells, i.e., VE-cadherin, CD31, and vimentin, in cultured HNSCC cell lines and solid HNSCC tumors, expand the cell plasticity continuum to include the endothelial-like phenotype which fulfills a distinct role from the EMT. Acquisition of endothelial characteristics by cancer cells enhances the VEGF-based autocrine-paracrine growth loop [10], facilitates cell invasion and promotes tumor-associated angiogenesis. As a component of the phenotypic state reciprocity, assumption of endothelial phenotype may occur concurrently with other phenotypic transitions to sustain a pro-proliferative, pro-invasive growth state for tumor progression through their combined effects.

While the expression pattern of vimentin in HNSCC cells is analogous to that in HUVECs, only a subpopulation of HNSCC cells express vimentin. In contrast, the location of CD31 staining was cell origin specific. The endothelial lineage cells (HUVEC) demonstrate classic membrane staining. Similar to the findings in human uterine squamous cell carcinomas [51], human HNSCC cells showed a cytosolic CD31 distribution. These data are consistent with assumption of other endothelial characteristics in HNSCC cell subpopulations. Furthermore, human HNSCC tumor samples also demonstrated both inter-patient and intra-tumor variations in expression of endothelial markers. For example, CD31 expression was highest in HNSCC cells adjacent to necrotic tumor whereas VE-cadherin was most prominent at the advancing invasive tumor margin. The heterogeneity present in HNSCC tumor cells e.g. high VEGF production by some cells with assumption of a mobile endothelial phenotype by others could benefit progression of the tumor as whole. These HNSCC phenotypic modulations appear to reflect tumor cell responses to changes in its dynamic microenvironment.

Similar to our findings, Sajithlal et al. [34] successfully identified and isolated tumor-derived endothelial-like cells (TDEC) from a variety of human cancer xenografts (breast,

prostate, and lung cancers). Those TDEC cells contain only human chromosomes and share variable commonalities with endothelial cells [34]. Sajithal et al. speculate that the transition from cancer cells to TDEC may be due to a transdifferentiation-like process and/or VM [34]. While our data demonstrated assumption of immunological and functional endothelial characteristics including tube-like structure formation in HNSCC cells and tumors, we failed to identify any blood cell-containing VM structures in our 24 separate HNSCC tumor specimens. These findings imply that the functional consequences following assumption of the endothelial phenotype may not be restricted to formation of VM network per se. The acquisition of enhanced mobility via cadherin switching in conjunction with facilitation of tumor progression by augmenting the HNSCC-VEGF growth loop provides a solid rationale for development of this epithelial to endothelial phenotypic transition.

In addition to tube-like structure formation on 3D cultures, internalization of low density lipoprotein is another unique functional characteristic attributed to endothelial cells [24, 25]. Perturbations in this “scavenger” function are speculated to contribute to the pathogenesis of atherosclerosis [24]. Accordingly, endocytosis of AcLDL is widely used as a screening assay to identify endothelial cells [25]. Our qualitative and quantitative results confirmed that HNSCC cells internalize AcLDL and that there are cell-line related differences in uptake capacity. Furthermore, while the scavenger receptors (SR-A and CD36) are associated with LDL uptake in macrophages and endothelial cells [27, 28], such receptors were not detected in HNSCC cells.

These data imply that HNSCC cells employ receptor-independent mechanisms such as pinocytosis to internalize AcLDL. Previous studies from our lab, which demonstrate the uptake of nanoparticles by HNSCC cells, support this premise [35]. As an important cholesterol carrier, LDL is crucial to maintain the cholesterol homeostasis in both normal endothelial cells and transformed tumor cells [36, 37]. Consequently, new cholesterol delivered into tumor cells via LDL internalization could preserve cell energy reserves by suppression of endogenous cholesterol production [38]. Furthermore, LDL delivered cholesterol could contribute an essential cell membrane component which is vital to support rapid tumor cell growth and division [38].

TGF- β 1 is known to induce the EMT via Smad and non-Smad pathways during both normal growth and carcinogenesis [39]. Relevant to HNSCC progression, previous studies have demonstrated that TGF- β 1 is overexpressed in human HNSCC tumors and adjacent tissues [40], and is associated with MMP-9 induction in HNSCC cells [41]. Furthermore, while TGF- β 1 upregulates VEGF production in human cancer cells [42], additional studies failed to show an association between VEGF secretion and microvessel density in HNSCC tumors [43]. These data suggest that TGF- β 1 stimulated HNSCC production of VEGF may fulfill other functions in addition to angiogenic induction e.g. an intracrine growth factor. Our data revealed that TGF- β 1 treatment significantly augmented AcLDL uptake in every HNSCC cell line, despite their baseline differences in AcLDL uptake. Interestingly, the cell line that showed the lowest baseline and TGF- β 1 stimulated AcLDL uptake (SCC15) was also the most TGF- β 1 responsive with regard to cadherin switching and invasion. These data indicate that TGF- β 1 affects multiple pathways and that the relative extent of transition to an endothelial phenotype is contingent on the responsiveness of the heterogeneous HNSCC cell subpopulations.

In order to clarify the underlying mechanism responsible for TGF- β 1 induced endothelial phenotypic transition, TGF- β 1's effect on expression of endoglin protein in HNSCC cells was investigated. Endoglin, which is a homodimeric membrane glycoprotein that functions as an auxiliary receptor for the TGF- β family, binds a number of TGF- β superfamily members including TGF- β 1, TGF- β 3, activin-A, BMP-7 and BMP-2 in association with

TGF- β type I and type II receptors [44, 45]. While endoglin is typically expressed in vascular endothelial cells, macrophages, fibroblasts and stromal cells, endoglin expression has also been identified in transformed epithelial lineage cells such as prostate cancer cells [46] and metastatic breast cancer cells [47]. Endoglin overexpression was found to be associated with enhanced invasive phenotype of human breast cancer cells [47]. Also, membrane-bound endoglin could be shed and released into stroma as a soluble form which is associated with the development of poorly differentiated carcinoma [29, 30]. Our data show that the baseline levels of endoglin in HNSCC cells are relatively low compared to HUVECs. Exposure to TGF- β 1, however, significantly increased endoglin levels in CAL27 and SCC15 cell lysates. While other studies have detected endoglin in human cancer cells [46, 47], these data were reported as relative amounts, making direct comparisons to HNSCC endoglin levels impossible. Notably, SCC15 cells are most responsive to TGF- β 1 with regard to cadherin switching and cell invasion. In contrast, endoglin levels in SCC9 cells, which are refractory to TGF- β 1 modulation of cadherins and invasion, remained negligible regardless of TGF- β 1 treatment.

It is well established that, in addition to its crucial role in cancer progression, the EMT is also an essential process for normal growth and development [17]. Our results, which showed the expression of vimentin in ulcerated epithelium, support the role of EMT in facilitating epithelial cell migration necessary for ulcer healing. The absence of CD31 and VE-cadherin in normal and ulcerated oral epithelium and *in vitro* cultured HOKs together with the negative results of tube-like structure formation and AcLDL uptake shown by HOKs, indicate that the most extensive phenotypic plasticity is more associated with transformed malignant cells. Our *in vitro* cell data was validated by IHC studies on HNSCC tumor tissues which showed endothelial HNSCC cells were predominantly located at the advancing end (including metastases) of the tumor. While normal physiological process such as wound healing may recruit a transient pro-migratory phenotypic transition like EMT, a sustained pro-proliferative and pro-migratory growth state which involves multidirectional phenotypic transitions (including epithelial-endothelial phenotype reciprocity) appears essential for malignant tumor progression.

We have previously reported that VEGFR1 and VEGFR2 are present in cultured HNSCC cells and VEGF enhanced HNSCC cell proliferation and invasion [10]. Binding of VEGF to its cognate tyrosine kinase receptors (VEGFRs) elicits activation of a series of intracellular signaling cascades including Erk and Src pathways, resulting in expression of variable downstream genes associated with proliferation and migration [48]. VEGF-mediated Erk and Src activation thus indicates the endothelial or endothelial-like phenotype of target cells. These current data, which show that VEGF challenge initiates VEGF-receptor mediated intracellular signaling in CAL27 cells, substantiate our previous HNSCC-VEGF observations. While similar studies were conducted on the all three HNSCC lines, the CAL27 cells were the most responsive to exogenous VEGF. Previous studies [10, 49] have shown that most HNSCC cell lines produce exceptionally high levels of VEGF resulting in these cells being refractory to exogenous VEGF. In contrast, CAL27 cells (also referred to as SCC2095) produce relatively lower VEGF levels and thereby retain responsiveness to exogenous VEGF without the need for siRNA blocking of endogenous VEGF. The fact that HNSCC cells not only serve as target of VEGF, but also respond to angiostatic agent endostatin substantiates the premise that HNSCC tumors and isolated cells contain at least a cellular subpopulation with endothelial characteristics.

Previous and current data from our lab and others demonstrated that the angiostatic agent, endostatin, interfered with HNSCC cell migration and HNSCC-VEGF crosstalk in an analogous fashion to its effects on endothelial cells [8, 9]. Without the potential for clinical implications, the recognition of an endothelial transition in HNSCC cells is merely an

interesting observation. The clinical relevance of these findings, however, lies with regard to implementation of future treatments. Notably, introduction of compounds that target angiogenesis-associated pathways, e.g., bevacizumab, sorafenib, have improved the prognosis and survival of HNSCC patients [6, 50]. These findings imply that the positive clinical effects of these agents likely reflect both their angiostatic and antitumorigenic properties and establishes the precedent to identify additional, more selective agents capable of concurrently inhibiting both the activated endothelial cells as well as the endothelial HNSCC cells necessary for tumor associated angiogenesis and metastasis, respectively.

Conclusions

Our data demonstrate that HNSCC tumor cells express markers traditionally regarded as endothelial cell specific with the highest expression detected at the leading edges of the tumor nests (VE-cadherin) and peri-necrosis regions (CD31 and vimentin). Cultured HNSCC cells recapitulate these findings as demonstrated by expression of endothelial markers, and also show functional endothelial characteristics such as AcLDL uptake and responsiveness to VEGF and endostatin. Furthermore, the well-established tumor phenotypic modulator, TGF- β 1, enhances these endothelial characteristics and augments invasiveness in HNSCC cell subpopulations. Collectively, these findings imply that assumption of an endothelial phenotype by at least a subpopulation of cells facilitates HNSCC progression by enhancing cell mobility and establishing a pro-angiogenic and pro-proliferative VEGF-mediated intracrine growth loop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institution of Health (NCI R01CA129609 NCI RC2CA148099 and NCI R01CA171329 to Dr. Susan R. Mallery, F30 DE02992 to Andrew S. Holpuch and T32 DE14320 to Byungdo B. Han). We thank Mary Marin and Mary Lloyd, our histotechnologists, for their assistance with tissue specimen preparation.

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Figure 1. HNSCC cells express endothelial-associated proteins. (A) Immunocytochemistry staining of the vascular endothelial intercellular adhesion molecule VE-cadherin on HUVECs and HNSCC cells. HUVECs served as the positive control. (B) Western Blots demonstrated the presence of VE-cadherin protein in HNSCC cells and also revealed a cell-line dependent level of expression. The additional lower molecular weight band shown in HUVEC lysate likely represents a proteolytic fragment of VE-cadherin during apoptosis of a subpopulation of HUVECs [22]. Ratios of VE-cadherin band density versus matched β -actin band density are presented at the bottom. Normal HOKs did not express VE-cadherin by either ICC staining (data not shown) or Western Blot (analyzed on a separate gel).

Figure 2.

HNSCC cells express vimentin and CD31 proteins. Left four panels: Double staining of endothelial/mesenchymal intracellular intermediate filament vimentin (red) and its epithelial counterpart cytokeratin 6 (green). While HUVECs showed universal expression of vimentin (A), only a subpopulation of cells in each HNSCC cell line demonstrated vimentin positive staining (C, E, G). Cytokeratin 6 (green) was exclusively expressed in HNSCC cells (C, E, G). Right four panels: Immunocytochemistry staining of CD31. HUVECs exhibited classic membrane expression of CD31 (B). A subpopulation of HNSCC cells demonstrated cytosolic staining of CD31 (D, F, H).

Figure 3.

HUVECs and HNSCC cells form tubular networks on Matrigel. Log growth HUVEC, HOK and HNSCC cells were added to Matrigel pre-coated 24-well plates and then incubated for 17 hrs. The HUVEC cultures generated delicate tubular structures whereas the HNSCC cells aligned in a coarser network. HOK cells, however, remained in a dispersed monolayer and did not display the same cell interaction patterns.

Figure 4.

HNSCC cells internalize AcLDL, which is enhanced following TGF- β 1 challenge. (A) Qualitative studies. Subconfluent HUVECs, which served as the positive control, HOKs, or HNSCC cells were exposed to 10 μ g/ml Alexa Fluor 488 conjugated AcLDL (green dots) for 4 h. Extracellular Alexa Fluor 488 signals were quenched with 0.08% Trypan Blue followed by two washes with PBS. CD31 and E-cadherin were stained (red) to delineate HUVECs or HOKs and HNSCC intercellular boundaries respectively. Nuclei were counterstained with DAPI (blue). Normal oral mucosa was included as a positive control for E-cadherin. (B) Quantitative studies. HNSCC cells were treated with 0 (control) or 10 ng/ml TGF- β 1 (24 h) followed by Alexa Fluor 488 conjugated AcLDL incubation and Trypan Blue quenching. AcLDL internalization data were then obtained and quantified relative to an assay specific standard curve. Data were normalized to ng LDL per 10⁵ cells. HUVECs (n =

8) and the transformed monocyte cell line U937 ($n = 3$) were employed as positive control populations.

Figure 5.

TGF- β 1 induces an endothelial phenotype in HNSCC cells. (A) SCC15 cells were treated with 0 (control) or 10 ng/ml TGF- β 1 for 24 h prior to immunocytochemistry staining of epithelial phenotypic intercellular adhesion molecule E-cadherin and three endothelial markers i.e. CD31, vimentin and VE-cadherin. Microscopic field-matched images of nuclei (labeled with DAPI) were included to demonstrate the comparable cell densities between the control and TGF- β 1 treated groups. (B) Endoglin ELISAs were conducted on cell lysates of HUVECs (positive control) and three HNSCC cell lines either pretreated with 10 ng/ml TGF- β 1 or control for 48 h. Endoglin levels were normalized to pg per mg total protein, data expressed as mean $\pm$ S.E.M. Y axis was sectioned due to the great difference of endoglin levels between HUVECs and HNSCC cells. (C) SCC9 and SCC15 cells were exposed to 0 (control) or 10 ng/ml TGF- β 1 for 24 h prior to the 48 h cell invasion assay. Invaded cell

numbers were determined relative to the cell line specific standard curves. Error bars represent standard error of mean, $n = 5$.

Figure 6.

Endothelial associated proteins are also present in HNSCC tumors. Five intact normal oral epithelium samples, 10 ulcerated, non-neoplastic oral mucosal biopsies and 24 HNSCC tumor tissues respectively underwent IHC staining for pancytokeratin and three traditionally mesenchymal and/or endothelial markers i.e. vimentin, CD31 and VE-cadherin. Images demonstrated in each column represent the same microscopic field of a same tissue specimen. Normal, intact oral epithelium is characterized by extensive cytokeratin positivity, with vimentin, CD31 and VE-cadherin positivity restricted to connective tissue cells (vimentin) and endothelium (CD31 and VE-cadherin). In contrast, oral epithelium adjacent to an ulcer demonstrates vimentin positivity. Primary HNSCC tumors showed intense keratin positivity, moderate to high vimentin and CD31 expression and modest VE-cadherin. Metastatic tumor cells and advancing tumor edges demonstrated higher levels of “endothelial” markers. Interestingly, many “endothelial marking” tumor cells retained dual expression of cytokeratin. In the peri-necrotic tissue zones, CD31 positivity was observed in endothelial cells, some infiltrating inflammatory cells and the HNSCC cells.

A VEGF induced phosphorylation of Erk1/2 and Src in HNSCC cells

B Endostatin inhibits VEGF-induced HNSCC intracellular signaling

Figure 7.

VEGF activates comparable signaling pathways in HNSCC and endothelial cells. (A) VEGF induces a rapid phosphorylation of Erk1/2 and Src. 24 h serum deprived CAL27 cells were challenged with 50 ng/ml VEGF for 0, 1, 5, 10, 20, 30 or 60 min. Cell lysates were then obtained for Western Blot analyses of both total and phosphorylated levels of Erk1/2 and Src. (B) Endostatin (10 μ g/ml) pretreatment diminished CAL27 intracellular signaling, as manifested by a reduction in VEGF-mediated phosphorylation of Erk1/2 and Src relative to control cultures. Notably, a 60 min endostatin pretreatment ablated CAL27 responsiveness to VEGF-induced phosphorylation. Intensity ratios of phosphorylated and matched total proteins were presented at the bottom of each section.

Table I

Patient demographics for immunohistochemistry study.

Sample# [†]	Primary Site	Diagnosis	Sex	Age	Tumor source	pTNN [*]	Stage [*]	Differentiation
MP001	larynx	Metastatic SCC	M	52	primary site	TxN2cMx	IVA	Poor
MP002	tongue	SCC	F	48	primary site	T3N2Mx	IVA	Moderate
MP003	oral cancer mets to lung	metastatic SCC	M	52	metastatic tumor	TxNxM1	IVA	Poor
MP004	oral cancer mets to neck	metastatic SCC	M	58	metastatic tumor	TxN1Mx	III	Moderate-Poor
MP005	larynx	SCC	M	67	primary site	T3N1Mx	III	Moderate-Poor
MP006	base of tongue	metastatic SCC	M	44	primary site	TxN2Mx	IVA	Moderate
MP007	right parotid	SCC	M	74	primary site	T3N0Mx	III	Moderate
MP008	oral cancer mets to neck	SCC	M	73	metastatic tumor	TxN1Mx	III	Poor
MP009	left tonsil	SCC	M	67	primary site	T1N2bMx	IVA	Poor
MP010	right tongue	SCC	F	73	primary site	T3N2aMx	IVA	Well
MP011	palate	SCC	M	59	primary site	T2N0Mx	II	Well-Moderate
MP012	larynx	SCC	M	53	primary site	T3N0Mx	III	Well-Moderate
MP013	tongue	SCC	F	82	primary site	TxN2Mx	IVA	Moderate
MP014	larynx	SCC	M	64	primary site	T3N2cMx	IVA	Moderate
MP015	floor of mouth	SCC	M	61	primary site	T4aN2cMx	IVA	Moderate
MP016	base of tongue	metastatic SCC	F	66	metastatic tumor	TxN2cMx	IVA	Poor
MP018	metastatic tongue cancer	metastatic SCC	M	73	primary site	TxN1Mx	III	Moderate-Poor
MP019	tonsil	metastatic SCC	M	45	metastatic tumor	T2N2bMx	IVA	Poor
MP021	larynx	SCC	F	75	primary site	T4aN0Mx	IVA	Moderate
MP022	tongue	SCC	F	35	primary site	T2N0Mx	II	Moderate
MP024	tongue	SCC	M	51	primary site	T4aN2bMx	IVA	Moderate
MP025	larynx	SCC	F	58	primary site	T2NxMx	II	Moderate
MP026	oral cavity	SCC	F	79	primary site	T3N0Mx	III	Moderate
MP027	tonsil	metastatic SCC	M	59	metastatic tumor	T2N2bMx	IVA	Moderate

* Tumor-Node-Metastasis (TNM) Classification and Clinical Staging was determined according to American Joint Committee on Cancer (AJCC) Cancer Staging Manual (7th ed. 2010.).

** Metastatic

*** Squamous cell carcinoma

⁷ Samples MP017, MP020, MP023 were excluded from this study due to their infiltrative tumor growth patterns which precluded delineation of tumor tissue from stroma

Table II

Percentage of cases demonstrating positive staining of VE-cadherin, CD31 and Vimentin

Total	pN Stage		Clinical Stage	
	N0 (8) [*]	N>0 (16)	II (3)	>II (21)
VE-cadherin	37.5% (3)	43.8% (7)	33.3% (1)	47.6% (10)
CD31	100% (8)	81.3% (13)	100% (3)	85.7% (18)
Vimentin	87.5% (7)	93.8% (15)	66.7% (2)	95.2% (20)

^{*} Numbers listed in the parentheses () represent the total cases fit into each subcategory.