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Endothelial derived factors inhibit anoikis of head and neck cancer stem cells

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Abstract

Recent evidence demonstrated that cancer stem cells reside in close proximity to blood vessels in human head and neck squamous cell carcinomas (HNSCC). These findings suggest the existence of a supporting perivascular niche for cancer stem cells.

Objective—The purpose of this study was to evaluate the effect of endothelial cell-secreted factors on the behavior of head and neck cancer stem-like cells (HNCSC).

Materials and methods—HNCSC were identified by sorting UM-SCC-22A (cell line derived from a primary squamous cell carcinoma of the oropharynx) and UM-SCC-22B (derived from the metastatic lymph node of the same patient) for CD44 expression and ALDH (aldehyde dehydrogenase) activity. HNCSC (ALDH+CD44+) and control (ALDH–CD44–) cells were cultured in ultra-low attachment plates in presence of conditioned medium from primary human endothelial cells.

Results—ALDH+CD44+ generated more orospheres than control cells when cultured in suspension. The growth factor milieu secreted by endothelial cells protected HNCSC against anoikis. Mechanistic studies revealed that endothelial cell-secreted vascular endothelial growth factor (VEGF) induces proliferation of HNCSC derived from primary UM-SCC-22A, but not from the metastatic UM-SCC-22B. Likewise, blockade of VEGF abrogated endothelial cell-induced Akt phosphorylation in HNCSC derived from UM-SCC-22A while it had a modest effect in Akt phosphorylation in HNCSC from UM-SCC-22B.

Conclusion—This study revealed that endothelial cells initiate a crosstalk that protect head and neck cancer stem cells against anoikis, and suggest that therapeutic interference with this crosstalk might be beneficial for patients with head and neck cancer.

Keywords

Head and neck squamous cell carcinoma; Perivascular niche; Angiogenesis; Tumor microenvironment; Metastasis

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INTRODUCTION

Mounting evidence suggest that stem cells are involved in the pathogenesis of several cancers, including those of the head and neck.¹⁻⁴ Stem cells and tumor cells have many features in common. Both have the potential for self-renewal and/or differentiation, and both tend to be long-lived cells.⁵ Moreover, tumor cells and stem cells are resistant to apoptosis and have enhanced telomerase activity.⁶ Since cancer stem cells have been identified as the driving force in tumors,⁵ it has become apparent that they must be eradicated to enhance the survival of cancer patients.⁷ Cancer stem cells sorted from primary tumors such as pancreatic adenocarcinomas³ or head and neck squamous cell carcinomas (HNSCC)⁸ are able to self-renew, and to produce tumors when implanted in very low numbers in immunodeficient mice. Based on the demonstrated similarities between normal tissue stem cells and cancer stem cell,⁹ it has been postulated that cancer stem cells may also exist within a supportive niche. Indeed, cancer stem cells are found in close proximity to blood vessels in human head and neck tumors, which is suggestive of a perivascular niche in these tumors.⁸ Understanding the mechanisms and biological consequences of the crosstalk between cancer stem cells and endothelial cells may reveal new molecular targets for head and neck cancer treatment.

Soluble factors secreted by endothelial cells have been shown to be an essential component of the normal stem cell niche by promoting self-renewal and inhibiting differentiation of neural stem cells.¹⁰ Indeed, a perivascular niche for brain tumor stem cells has been identified, where endothelial cells appear to play an important role in stem cell mediated initiation and progression of tumors.^{11,12} We have recently shown that endothelial cell-initiated signaling induce Bmi-1 expression and self-renewal of head and neck cancer stem cells, and that selective ablation of tumor-associated endothelial cells with a caspase-based artificial death switch correlates with a decrease in the cancer stem cell fraction.⁸ These findings raise the intriguing possibility that head and neck cancer stem cells rely on interactions with endothelial cells to maintain their stemness, and consequently, to sustain their role in the pathobiology of the tumor.

It is known that endothelial cells have a significant impact on head and neck tumor growth by secreting factors (*e.g.* VEGF) that affect tumor cell proliferation and tumor cell phenotype.¹³ Endothelial derived factors (*e.g.* IL-6, EGF) induce activation of key signaling molecules (*e.g.* STAT3, ERK) in head and neck tumor cells enhancing their motility and inhibiting anoikis.^{14,15} These studies were performed with unsorted HNSCC cell lines. Here, we evaluated the effect of endothelial cell-secreted factors, particularly VEGF, on the phenotype (*i.e.* proliferation and survival) of cancer stem cells sorted from primary and from metastatic HNSCC.

MATERIALS AND METHODS

HNCSC sorting and culture

A cell line generated from the surgical removal of a primary tumor localized in the hypopharynx (UM-SCC-22A), and the metastatic cell line (UM-SCC-22B) derived from a metastatic lymph node from the same patient¹⁶ were used in this study. The identity and purity of these cell lines were confirmed by short tandem repeat (STR) profiling. Head and neck cancer stem cell-like cells (HNCSC) were identified by cell sorting for CD44 expression and ALDH (aldehyde dehydrogenase) activity, as we showed previously.⁸ Briefly, the Aldefluor kit (StemCell Technologies; Durham, NC, USA) was used to identify cells with high ALDH enzymatic activity, while anti-CD44 antibody (BD Pharmingen; Franklin Lakes, NJ, USA) was used to sort for CD44 expression in a FACSDiVA Cell

Sorter (BD Biosciences; Mountain View, CA, USA). Cells were also gated for 7AAD negative cells to eliminate dead cells or debris. After sorting, CD44+ALDH+ (HNCSC) and CD44-ALDH- (control cells) were cultured in suspension in ultra-low attachment plates (Corning; New York, NY, USA), as described^{8,17} using low glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen).

Proliferation assays

Cells (5×10^3) were seeded in 96-well ultra low-attachment plates. After 24 hours, CD44+ALDH+, CD44-ALDH- were exposed to endothelial cell conditioned medium (EC CM). Briefly, conditioned medium from primary human dermal microvascular endothelial cells (HDMEC, Lonza; Walkersville, MD, USA) was prepared in serum-free endothelial basal medium (EBM; Lonza), filtered and concentrated 10x, as we described.¹³ Cells were fed with 10x HDMEC-CM (1 part) reconstituted in DMEM/10% FBS (9 parts). As a positive control, cells were treated with 50 ng/ml rhVEGF₁₆₅ (R&D Systems; Minneapolis, MN, USA). Alternatively, cells were pre-incubated with 1 µg/ml anti-VEGF or IgG isotype control (R&D Systems) for 1 hour at 37°C, and then incubated with EC-CM in presence of these antibodies, as described.¹³ As an additional control, we used unconditioned EBM (1 part) reconstituted in DMEM/10% FBS (9 parts). WST-1 cell proliferation assay kit (Roche; Mannheim, Germany) was used according to the manufacturer's instructions. Alternatively, cell density was determined using sulforhodamine B (SRB) method, as we described.¹⁴

Apoptosis assay

ALDH+CD44+ or control cells (1×10^5) were seeded in six-well ultra low-attachment plates, and exposed to EC-CM or EBM. After 24 hours, cells were retrieved, exposed to a hypotonic solution of propidium iodide and analyzed by flow cytometry (FACSCalibur Cytometer, BD Biosciences) to determine the percentage of apoptotic cells (*i.e.* sub-G₀/G₁), as previously described.¹⁸

Orosphere assay

To evaluate the cells ability to grow in suspension as "orospheres",⁸ 1×10^5 ALDH+CD44+ or control cells were cultured in six-well plates. The number of orospheres was determined with the ImagePro Plus 7.0 software (Media Cybernetics; Silver Spring, MD, USA) using images taken at 20x after 7 days in culture.

Western Blots

ALDH+CD44+, ALDH-CD44- or unsorted cells (1×10^5) were plated in six-well plates and cultured under the conditions described above. Total protein was electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes that were exposed to 1:1,000 rabbit anti-human phospho-Akt (9271, Cell Signaling; Danvers, MA, USA); 1:1,000 rabbit anti-human Akt (9272, Cell Signaling); 1:1,000 rabbit anti-human VEGFR1, 1:1,000 rabbit anti-human VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA); 1:10,000 mouse anti-GAPDH (MAB374, Millipore; Billerica, MA, USA). Immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific; Rockford, IL, USA).

Statistical analysis

Data were analyzed by *t*-test or one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests. Statistical significance was determined at $P < 0.05$. These analyses were performed using the SigmaStat 2.0 software (SPSS; Chicago, IL, USA).

RESULTS

ALDH and CD44 identify cells with *in vitro* stem-like phenotype

To evaluate the capacity of ALDH and CD44 to identify cells with a stem-like behavior *in vitro*, we sorted UM-SCC-22A and UM-SCC-22B for these two markers and plated the cells in ultra-low attachment plates. We observed ALDH+CD44+ cells from both cell lines formed more orospheres ($P<0.05$) than ALDH-CD44- controls (Figure 1A, 1B).

Orospheres are characterized as “spheres” of cells derived from HNSCC that grow in suspension.⁸ Interestingly, ALDH-CD44- control cells sorted from the metastatic UM-SCC-22B formed less orospheres than the control cells from the primary tumor-derived cell line UM-SCC-22A (Figure 1B). Proliferation assays revealed that ALDH+CD44+ cells grow at a quicker rate than control ALDH-CD44- cells (Figure 1C).

Endothelial secreted factors enhance the survival of HNCSC

To evaluate the effect of the endothelial secreted growth factor milieu on the survival of HNCSC, we cultured ALDH+CD44+ and ALDH-CD44- cells in suspension under serum-free conditions. We observed that endothelial cell conditioned medium protected ALDH+CD44+ cells ($P<0.05$) sorted from the primary cell line (UM-SCC-22A) and metastatic cell line (UM-SCC-22B) against anoikis induced by lack of anchorage and serum starvation (Figure 2A). In contrast, the conditioned medium had a protective effect for ALDH-CD44- cells sorted from the UM-SCC-22A (Figure 2A), but not for ALDH-CD44- cells sorted from the UM-SCC-22B (Figure 2B) cell line.

VEGF secreted by endothelial cells enhances the proliferation of primary, not metastatic, HNCSC

We observed that the growth factor milieu secreted by endothelial cells induces the proliferation of all cell types evaluated here (Figure 3). In search for a mechanism for this response, we observed that endothelial secreted VEGF plays an important role in the proliferation of UM-SCC-22A (ALDH+CD44+ and ALDH-CD44-), since its blockade with anti-VEGF antibody abrogate the inductive effect of the conditioned medium (Figure 3A, 3B). Surprisingly, the trends were significantly different when metastatic UM-SCC-22B cells were evaluated (Figure 3C, 3D). In this case, blockade of VEGF from the conditioned medium did not have any effect on ALDH+CD44+ or ALDH-CD44- cells. Likewise, stimulation with recombinant human VEGF₁₆₅ also did not result in induction of cell proliferation.

Endothelial secreted VEGF induces phosphorylation of Akt in HNCSC

It is well known that the PI3k-Akt is a critical signaling pathway for the regulation of cell proliferation and cell survival in health and in disease.¹⁹ In search for a molecular mechanism to explain the results described above, we performed a series of studies to evaluate the effect of the endothelial cell growth factor milieu on the activation of the PI3k-Akt pathway. We observed that exposure of ALDH+CD44+ cells to endothelial cell conditioned medium induced potent and fast (*i.e.* within 15 minutes) phosphorylation of Akt (Figure 4A, 4C). In contrast, exposure of control (ALDH-CD44-) cells to the conditioned medium induced activation of Akt only in cells sorted from primary (UM-SCC-22A) cell line (Figure 4B), but not in cells sorted from the metastatic (UM-SCC-22B) cell line (Figure 4D). Blockade of VEGF from the conditioned medium abrogated Akt phosphorylation in ALDH+CD44+ cells from primary UM-SCC-22A (Figure 4A), and had a more modest effect on Akt phosphorylation in ALDH+CD44+ cells from metastatic UM-SCC-22B (Figure 4C) or in ALDH-CD44- cells sorted from the UM-SCC-22A (Figure 4B). In contrast, VEGF inhibition had no effect on Akt phosphorylation in control ALDH-CD44-

cells sorted from the metastatic UM-SCC-22B cell line (Figure 4D). Interestingly, the ALDH+CD44+ cells sorted from UM-SCC-22A present constitutive phosphorylation of Akt, while ALDH-CD44- cells do not (Figure 4A, 4B), suggesting that these two markers (ALDH and CD44) discriminate cells with divergent activation levels of this important signaling pathway. Both unsorted and sorted UM-SCC-22A and UM-SCC-22B express VEGFR1, but not VEGFR2 (Figure 4E). Notably, while endothelial cell conditioned media induced VEGFR1 expression in ALDH+CD44+ cells from both cell lines, the expression of the shorter VEGFR1 isoform was more pronounced in the UM-SCC-22A cells (Figure 4F).

DISCUSSION

The search for factors that play a role in the survival of cancer stem cells has tremendous therapeutic implications due to the recent observation that these cells appear to be the “drivers” of the progression several tumors, including head and neck squamous cell carcinomas.²⁰ Here, we demonstrated that endothelial cell-secreted factors induce the proliferation and enhance the survival of head and neck cancer stem cells *in vitro*. More specifically, endothelial secreted VEGF activates PI3k-Akt signaling and induces the proliferation of head and neck cancer stem cells. Collectively, these findings might begin to provide a mechanism for results reported recently by our laboratory that showed that stem cells are preferentially localized in perivascular niches in human head and neck squamous cell carcinomas.⁸

We have previously shown that endothelial cell secreted factors enhance xenograft tumor growth, migration of HNSCC cells, and protects these cells from anoikis.^{13,15} Here, we used a cell line derived from the primary tumor and one from the metastatic lymph node of the same patient to evaluate the effect of endothelial derived factors on the behavior of head and neck cancer stem-like cells. The rationale for this approach comes from the observation that stem-like cells can be successfully isolated from established cancer cell lines, and that such cells have become a widely accepted model.^{21,22} Indeed, HNSCC lines retain a small subpopulation of stem-like cells that are capable of generating cancer spheroids.^{23,24} In parallel studies using ALDH and CD44 to sort cells from either primary HNSCC or from cell lines, we have recently observed similar trends for survival and self-renewal of stem-like cells under low attachment conditions.⁸ Collectively, these studies suggest that while the use of cells retrieved from freshly resected human tumors might be considered ideal, the use of cells sorted from cell lines constitute a useful model for comparative analyses of the behavior of stem-like cells from primary or metastatic tumors.

Single marker ALDH or CD44 have been used to identify stem-like cells in various tumors including breast and head and neck cancers.^{1,2,25} Here, we observed that the combined use of ALDH and CD44 identifies two subpopulations of cells that have a markedly different behavior. HNCSC (identified as ALDH+CD44+) have higher proliferation rates, form more orospheres under low attachment conditions, and are more resistant to anoikis than control cells (ALDH-CD44-). We have also tried to generate orospheres with unsorted cells cultured under low attachment conditions. However, in this case most cells underwent cell death and very few (if any) orospheres were formed. These data, together with our recent demonstration that ALDH+CD44+ cells sorted from primary HNSCC are significantly more tumorigenic *in vivo* than control cells,⁸ provided strong support for the combined use of these two markers for the identification of cancer stem-like cells in head and neck tumors.

A key hypothesis tested in this study was that the molecular crosstalk initiated by endothelial cells enhances the stemness of head and neck tumor cells. We observed that endothelial secreted factors, activate the PI3k-Akt signaling, induce the proliferation, and protect ALDH+CD44+ cells against anoikis. The inductive effect of endothelial-derived

conditioned medium on the activation of Akt signaling was more potent than rhVEGF₁₆₅ by itself. This finding suggests that other factors secreted by endothelial cells also participate in signaling events that result in the phosphorylation of Akt in cancer stem-like cells. Notably, the PI3k/Akt pathway is a critical regulator of the survival of head and neck cancer cells,²⁶ and is a predictor of the response of head and neck cancer patients to therapy.²⁷ Collectively, these data suggest that patients with HNSCC might benefit from therapeutic disruption of the molecular crosstalk between endothelial cells and cancer stem cells. Indeed, we are currently performing experiments to evaluate therapeutic approaches to disrupt this crosstalk in preclinical models of head and neck cancer.

Interestingly, endothelial cell-secreted factors may have different roles on the biology of stem cells in primary tumors and metastatic tumors. In primary tumors, modeled here by the UM-SCC-22A cell line, endothelial cell-secreted VEGF induced proliferation of head and neck cancer stem-like cells. In contrast, in the metastatic UM-SCC-22B, the inductive effect of endothelial cell factors was independent from VEGF. This intriguing observation suggests that stem cells from the primary tumor may respond differently than stem cells from metastatic sites to anti-VEGF therapies. This is in line with reports showing differences in behavior of primary and metastatic head and neck tumors.^{28,29} Notably, a closer evaluation of the VEGFR1 expression patterns indicates that while the top VEGFR1 isoform is expressed in equal levels in ALDH+CD44+ cells sorted from either UM-SCC-22A or UM-SCC-22B that were exposed to endothelial cell conditioned medium, the lower VEGFR1 isoform is expressed in higher levels in the cells derived from the primary UM-SCC-22A. It is possible that individual VEGFR1 isoforms have unique signaling patterns as it has been reported for FGFR1, a closely related tyrosine kinase receptor.³⁰ This could explain (at least in part) the differences in response to endothelial cell-secreted factors that were observed here.

Our control experiments revealed some rather unexpected, yet reproducible, results. We observed that endothelial cell conditioned medium induced death of control ALDH-CD44- cell sorted from the metastatic UM-SCC-22B but not control cells sorted from the primary UM-SCC-22A. This may be correlated with the unexpected observation that endothelial cell conditioned medium did not induce phosphorylation of Akt in the ALDH-CD44- cells sorted from the metastatic UM-SCC-22B, while it did induce Akt signaling in the control cells derived from UM-SCC-22A. The finding that the ALDH-CD44- cells retrieved from the UM-SCC-22A (but not from the UM-SCC-22B) cell line are more anoikis-resistant than the ALDH+CD44+ is also intriguing. The diversity of mechanisms used by cells to interpret signals and regulate anoikis³¹ is a potential explanation for the responses found here. Another possible explanation is that these are cells sorted from cell lines that have been cultured *in vitro* for many passages, which might have somewhat affected their anoikis resistance. Our laboratory is currently performing experiments to understand the mechanisms underlying these rather surprising observations.

In summary, we report here that the crosstalk initiated by endothelial cells has a profound effect on key aspects of the pathogenicity of head and neck cancer stem cells. We have also observed that the stem-like cells from a primary tumor model respond differently to the factors secreted by endothelial cells, as compared to the stem-like cells derived from a metastatic model. These findings exemplify complexities involved in the biology of cancer stem cells, and suggest a potential mechanism to explain differences in response to therapy frequently observed in primary and metastatic tumors of the same patient. Improved understanding of the biology of the cancer stem cells will likely contribute to the development of mechanism-based therapies for head and neck cancer patients.

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Figure 1.

ALDH and CD44 identify cells with stem-like phenotype *in vitro*. **(A)** Representative phase contrast images of orospheres developed from ALDH+CD44+ (cancer stem-like cells) or ALDH-CD44- (control cells) sorted from the primary tumor-derived (UM-SCC-22A) or from the metastatic lymph node-derived (UM-SCC-22B) cell lines. Cells were cultured in ultra-low attachment plates for 7 days. **(B)** Graph depicting the quantification of the number of orospheres. Statistical significance was determined at $P < 0.05$ and are indicated by different low case letters. **(C)** Graph depicting the proliferation of ALDH+CD44+ or ALDH-CD44- cells, as determined by the WST-1 assay.

Figure 2.

Endothelial cell-secreted factors enhance the survival of HNCSC. **(A,B)** Graph depicting the percentage of apoptotic cells, as determined by propidium iodide staining followed by flow cytometry. ALDH⁺CD44⁺ (cancer stem-like cells) or ALDH⁻CD44⁻ (control cells) sorted from the primary tumor-derived (UM-SCC-22A) or from the metastatic lymph node-derived (UM-SCC-22B) cell lines. Cells were cultured either in serum-free conditioned medium collected from primary endothelial cells (EC CM) or in serum-free endothelial basal medium (EBM) in ultra-low attachment plates for 24 hours. Asterisks depict $P < 0.05$.

Figure 3. VEGF secreted by endothelial cells enhances the proliferation of primary, not metastatic, HNCSC. (A–D) Graph depicting the proliferation of ALDH+CD44+ or ALDH-CD44- cells, as determined by the WST-1 assay. Cells were cultured in serum-free conditioned medium collected from primary endothelial cells (EC CM) supplemented with 1 μ g/ml anti-VEGF antibody or non-specific isotype-matched IgG (control) in ultra-low attachment plates for 48 hours. As additional controls, we evaluated cells cultured in serum-free endothelial basal medium (EBM) supplemented (or not) with 50 ng/ml rhVEGF₁₆₅.

Figure 4.

Endothelial secreted VEGF induces phosphorylation of Akt in HNCSC. **(A–D)** Western blots depicting the expression of total and phosphorylated Akt in ALDH+CD44+ or ALDH-CD44- cells sorted from UM-SCC-22A or UM-SCC-22B. Cells were cultured in serum-free conditioned medium collected from primary endothelial cells (EC CM) supplemented with 1 μ g/ml anti-VEGF antibody or non-specific isotype matched IgG control in ultra-low attachment plates. As additional controls, we evaluated cells cultured in presence of EC CM without IgG for 60 minutes, or in presence of serum-free endothelial basal medium (EBM) supplemented (or not) with 50 ng/ml rhVEGF₁₆₅ during all time points. **(E)** Western blots depicting the expression of VEGFR1 and VEGFR2 in unsorted UM-SCC-22A and UM-SCC-22B cells, using primary human endothelial cells (HDMEC) as controls. **(F)** Western blot to evaluate the effect of EC CM on the expression of VEGFR1 in ALDH+CD44+ UM-SCC-22A and UM-SCC-22B cells, using unsorted HDMEC as controls.