Endothelial Cell-Initiated Signaling Promotes the Survival and Self-Renewal of Cancer Stem Cells

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Abstract

Recent studies have demonstrated that cancer stem cells play an important role in the pathobiology of head and neck squamous cell carcinomas (HNSCC). However, little is known about functional interactions between head and neck cancer stem-like cells (CSC) and surrounding stromal cells. Here, we used aldehyde dehydrogenase activity and CD44 expression to sort putative stem cells from primary human HNSCC. Implantation of 1,000 CSC (ALDH+CD44+Lin–) led to tumors in 13 (out of 15) mice, whereas 10,000 noncancer stem cells (ALDH–CD44–Lin–) resulted in 2 tumors in 15 mice. These data demonstrated that ALDH and CD44 select a subpopulation of cells that are highly tumorigenic. The ability to self-renew was confirmed by the observation that ALDH+CD44+Lin– cells sorted from human HNSCC formed more spheroids (orospheres) in 3-D agarose matrices or ultra-low attachment plates than controls and were serially passaged in vivo. We observed that approximately 80% of the CSC were located in close proximity (within 100-μm radius) of blood vessels in human tumors, suggesting the existence of perivascular niches in HNSCC. In vitro studies demonstrated that endothelial cell-secreted factors promoted self-renewal of CSC, as demonstrated by the upregulation of Bmi-1 expression and the increase in the number of orospheres as compared with controls. Notably, selective ablation of tumor-associated endothelial cells stably transduced with a caspase-based artificial death switch (iCaspase-9) caused a marked reduction in the fraction of CSC in xenograft tumors. Collectively, these findings indicate that endothelial cell-initiated signaling can enhance the survival and self-renewal of head and neck CSC. Cancer Res 70(23): 9969–78. ©2010 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer type, accounting for more than 500,000 new cases each year in the world (1). The integration of platinum-based chemotherapy to the curative management of HNSCC resulted in an improvement in the control of local-regional disease and enhanced organ preservation (2). However, as the control of local-regional disease improved, the incidence of distant metastatic disease has risen (3, 4). As a result, the overall survival rate for patients with HNSCC has not improved significantly over the past 30 years and continues to be one of the lowest among the major cancer types. This clinical observation suggests that by creating a nonfavorable local environment for head and neck tumor cells with current therapies, these cells acquire a more aggressive phenotype leading to distant metastasis. Better understanding of the pathobiology of HNSCC is urgently needed for the development of more effective therapies.

Cancer stem cells (CSC) constitute a subpopulation of cells that are multipotent, self-renewing, and capable of generating the entire heterogeneous population seen in tumors (5–8). CSC are believed to “drive” tumorigenesis of some cancer types, including breast and head-and-neck tumors (9–11). This implies that the successful growth of a metastasis of tumors that follow the CSC model requires that at least 1 CSC resists to therapy (12). Notably, CSC are slow-dividing cells that are capable of resisting to current therapies for cancer (13).

Stem cells and CSC are frequently found in unique micro-environments called the "niche" (14, 15). Cell-to-cell interactions through direct contact or secreted factors support the survival and maintain the stemness of stem cells in cancer and in normal tissues (16). Perivascular niches have been identified in neural stem cells (17–19) and neural tumors (20). However, it is not known if the stem cells of head and neck tumors are localized in close proximity to blood vessels and depend on interactions with the cellular components of vascular niches for their survival and stemness.
Head and neck cancer stem cells (HNCSC) were first identified using CD44 (9), a marker of stem cells in epithelial tumors (21, 22). Aldehyde dehydrogenase (ALDH), an enzyme found to be highly active in stem cells of various origins (23–25), was recently used to identify stem cells in HNSCC (26). Here, we utilized ALDH1 and CD44 to identify a subpopulation of cells that exhibit several properties of CSC, including self-renewal and capacity to regenerate heterogeneous tumors. Analysis of human HNSCC demonstrated that the majority of the CSC are located in close proximity to blood vessels. Using 3-dimensional (3-D) models in vitro, we showed that endothelial cell-secreted factors promote proliferation and self-renewal of HNCSC along with increased expression levels of Bmi-1. Notably, selective ablation of tumor-associated endothelial cells with a caspase-based artificial death switch resulted in a significant decrease in the number of CSC in vivo. Collectively, these data unveil the functional interdependency of cancer stem cells and vascular endothelial cells in head and neck tumors, and show proof-of-principle evidence that therapeutic targeting of tumor blood vessels reduces the number of CSC.

Materials and Methods

Cell culture

HNSCC cell lines (UM-SCC-1, UM-SCC-74A, UM-SCC-74B, UM-SCC-17A, UM-SCC-17B, UM-SCC-11B; gift from Dr. Carey, University of Michigan, Ann Arbor, Michigan) were cultured in Dulbecco’s modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Grand Island, NY), and human dermal microvascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) in endothelial cell growth medium-2 (Lonza, Walkersville, MD). Fluorescence-activated cell sorting (FACS) cells were cultured in low glucose DMEM, 10% FBS (Invitrogen), and 100 U/mL penicillin–streptomycin (Invitrogen) or 100 U/mL antibiotics antimycotic solution (AA) (Sigma, St. Louis, MO) in ultra-low attachment plates (Corning; New York, NY). Conditioned medium (CM) from HDMEC was collected in serum-free DMEM from 24-hour cultures. HDMEC stably transduced with iCasparase-9 (HDMEC–iCasparase-9) were generated as described (27). The identity of all tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility.

Head and neck cancer stem cell sorting

Informed consent was obtained from patients undergoing surgery for removal of HNSCC. Tumor specimens were collected within 30 minutes post-surgery and transported in DMEM low glucose, 10% FBS, and AAA at 4°C. Within 12 hours, the tumors were cut into small pieces and minced with a sterile scalpel until they could pass through a 25-mL pipette tip. They were suspended in a 9:1 mixture of DMEM-F12 (Hyclone, Waltham, MA) containing collagenase and hyaluronidase (Stem Cell Technologies; Vancouver, BC, Canada). The mixture was incubated at 37°C for 1 hour and passed through a 10-mL pipette every 15 minutes for mechanical dissociation. Cells were filtered through a 40-μm nylon mesh (BD Falcon; Franklin Lakes, NJ), washed with low glucose DMEM containing 10% FBS, and centrifuged at 800 rpm for 5 minutes. Single cell suspensions obtained from primary specimens (as well as from cell lines or xenografts) were washed, counted, and resuspended at 1 × 10^6 cells/mL PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high ALDH activity. Briefly, the cells were suspended with activated Aldefluor substrate (BODIPY-aminoacetate) or the negative control (diethylaminobenzaldehyde, a specific ALDH inhibitor) for 45 minutes at 37°C. Then, the cells were exposed to anti-CD44 (clone G44-26BD; BD Pharmingen, Franklin Lakes, NJ) and lineage markers (i.e., anti-CD2, CD3, CD10, CD16, CD18; BD Pharmingen). Mouse cells were identified using anti-CD44 antibody (BD Biosciences; Franklin Lakes, NJ) and eliminated. Cells were cultured in ultra-low attachment plates overnight to allow for recovery from the flow sorting procedure. Next day, the cells were either implanted in mice or plated for in vitro experiments. Anti-CD31 (Biolegend, San Diego, CA), anti-HA (Sigma), and 7-aminoactinomycin (7-AAD; BD Pharmingen) were used in selected experiments. Propidium iodide staining followed by flow cytometry was used for the identification of apoptotic cells (i.e., sub-G0/G1 cells). Here and throughout this article, studies were done in triplicate specimens per condition and time point, and 3 experiments were performed to verify reproducibility of the data.

Confocal microscopy

Frozen sections were treated with peroxidase (Dako, Carpinteria, CA) and the antigen retrieval solution (Dako) was used for 45 minutes at 90°C. The anti-ALDH1 (BD Biosciences), CD44 (Abcam, Cambridge, MA), and Factor VIII (Neomarkers, Fremont, CA) were pre-labeled with Alexafluor 488 or 594 using the Zenon labeling kit (Invitrogen), and confocal imaging was performed using Zeiss confocal (Carl Zeiss, Thornwood, NY). Z-stacked images of 8 individual images were generated. The deconvolution was done using Autoquant (Media Cybernetics, Bethesda, MD) and the 3-D reconstruction completed using the Imaris Software (Bitplane AG, Zurich, Switzerland).

Colonization assay and “orospheres”

Colonization assays were performed in 3-D suspension cultures, as described (24, 28). Orospheres (i.e., spheroids of HNSCC-derived cells) were generated from 5 × 10^3 cells cultured in triplicate in ultra-low attachment plates (Corning). Alternatively, the cells were mixed with 0.2% agarose and layered on plates that were precoated with a layer of 0.4% agarose. The cells were maintained in low glucose DMEM containing or not CM from HDMEC at a ratio of 3:1. Orospheres generated in ultra-low attachment plates were mechanically dissociated into single cell suspensions and replated to generate secondary and tertiary cultures.

SCID mouse model of human tumor angiogenesis

Xenograft tumors vascularized with functional human microvessels were generated in severe combined immunodeficient (SCID) mice (CB 17 SCID; Taconic, Germantown, NY), as described (29). Briefly, 1,000 CSC (ALDH+CD44+Lin−) or 10,000 noncancer stem cells (NCSC; ALDH−CD44−Lin−) were seeded with HDMEC for a total of 1 × 10^6 cells in poly-L-lactic
acid (Medisorb, Nicosia, Cyprus) biodegradable scaffolds. Bilateral scaffolds were implanted subcutaneously in the dorsum of each mouse. Mice were monitored daily for tumor growth for 6 months or until the volume of the tumor reached $0.85 \text{ cm}^3$. Alternatively, mice received scaffolds containing $9.99 \times 10^5$ HDMEC–iCaspase-9 and $1 \times 10^5$ CSC, or controls. Twenty-four days after transplantation of the scaffolds, mice received daily intraperitoneal injections of $2 \text{ mg/kg}$ AP20187 (ARIAD, Cambridge, MA) for 4 days to activate iCaspase-9 and selectively ablate tumor blood vessels, as described (27).

**Statistical analyses**

Student $t$ test or 1-way ANOVA followed by post hoc analyses was performed using the SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at $P < 0.001$ (unless otherwise specified).

**Results**

**ALDH$^+$CD44$^+$Lin$^-$ cells retrieved from primary head and neck squamous cell carcinomas are highly tumorigenic**

CD44 was used as the marker for stem cells in the study that demonstrated the existence of stem cells in HNSCC (9). However, a relatively large proportion of cells was found to be positive for CD44 in that study. Here, we examined the ability of ALDH activity and CD44 expression to identify cells that have a cancer stem-like phenotype. We observed a "gradient of stemness" ranging from the ALDH$^+$CD44$^+$ cells with the highest number of colonies in soft agar to ALDH$^-$CD44$^-$ and ALDH$^-$CD44$^+$ with the least number of colonies (Supplementary Fig. S1). In addition, we observed that ALDH and CD44 colocalize in a subpopulation of cells in primary human HNSCC (Supplementary Fig. S2). These data led us to adopt the combined use of ALDH and CD44 for identification of CSC throughout this work. The efficacy of tumor take was compared when putative CSC (ALDH$^+$CD44$^+$Lin$^-$) or non-CSC (ALDH$^-$CD44$^-$Lin$^-$) were implanted in immunodeficient mice (Fig. 1A). Single cell suspensions were prepared from 4 patients with primary human HNSCC immediately after surgical resection (Supplementary Fig. S3). Viable cells were selected with 7-AAD (P1) and then gated in sequence for ALDH activity and CD44 expression, after elimination of lineage (Lin) cells (Fig. 1A). We observed that 1.78% of the cells were ALDH$^+$CD44$^+$Lin$^-$ (putative CSC) and 8.7% were ALDH$^-$CD44$^-$Lin$^-$ (NCSC) in a representative tumor (HN 10) (Fig. 1A). To evaluate the tumorigenicity of these cells, 1,000 ALDH$^+$CD44$^+$Lin$^-$ or 10,000 ALDH$^-$CD44$^-$Lin$^-$ (10-fold

**Figure 1.** Combination of ALDH and CD44 selects highly tumorigenic cells. A, schematic representation of the approach used for the testing of the tumorigenic potential of cells sorted from primary tumors. ALDH$^+$CD44$^+$Lin$^-$ cells were isolated from HNSCC and serially transplanted into immunodeficient mice to generate primary and secondary xenografts. Representative flow cytometry: Viable cells (P1) were isolated from a head and neck squamous cell carcinoma patient (HN 10) using 7AAD. Lineage cells (Lin) were eliminated and remaining cells were gated for positivity to ALDH (P5), using diethyliaminobenzaldehyde (ALDH inhibitor) as reference. ALDH-cells are found in P5. ALDH$^+$ and ALDH$^-$ cells were gated against CD44 to select ALDH$^+$CD44$^+$Lin$^-$ (P8 = 1.78%) and ALDH$^-$CD44$^-$Lin$^-$ (P7 = 8.7%). SSC-A, side-scatter area; FSC-A, forward-scatter area. B, graph shows the number of tumors generated by the implantation of 1,000 ALDH$^+$CD44$^+$Lin$^-$ or 10,000 ALDH$^-$CD44$^-$Lin$^-$ cells. The presence of a tumor was determined clinically by palpation over a period of 6 months (primary xenograft) and additional 6 months (secondary xenograft) and confirmed histologically on tumor retrieval. C, graph shows the volume of xenograft tumors (primary and secondary) obtained by the implantation of 1,000 ALDH$^+$CD44$^+$Lin$^-$ or 10,000 ALDH$^-$CD44$^-$Lin$^-$ cells sorted from human HNSCC into immunodeficient mice. *, $P < 0.001$. D, graph shows the percentage of putative head and neck cancer stem cells (ALDH$^+$CD44$^+$Lin$^-$) in primary tumors, and primary and secondary xenografts.
more cells) were coimplanted with human endothelial cells to generate human xenograft tumors vascularized with human blood vessels in immunodeficient mice, as described (29). Thirteen of 15 implants generated tumors (as determined by palpation and confirmed by histological analysis) in the ALDH+/CD44+Lin− group as compared with 2 of 15 of the ALDH−/CD44−Lin− group, demonstrating that the combination of ALDH and CD44 allowed for the selection of a highly tumorigenic subpopulation of cells (Fig. 1B). To evaluate their capacity of self-renewal, viable tumor xenografts were retrieved at 6 months or when their volume reached 850 mm³; processed into single cell suspensions, and serially transplanted to other mice. All implants containing ALDH+/CD44+Lin− cells generated secondary tumors, whereas none of the implants containing ALDH−/CD44−Lin− cells generated tumors (Fig. 1B). The volume of the primary and secondary xenograft tumors generated with ALDH+/CD44+Lin− cells was higher than with the ALDH−/CD44−Lin− cells (P < 0.001) at the end of the experimental period (Fig. 1C). Notably, the fraction of the putative CSC (ALDH+/CD44+Lin−) in the primary and secondary xenografts remained low and comparable to the fraction of these cells in the primary human tumors (Fig. 1D).

**Xenografts generated with ALDH+/CD44+Lin− cells resemble the primary tumors**

The histological organization of the primary and secondary xenograft tumors generated from the ALDH+/CD44+Lin− cells was comparable to the primary tumor from which these cells were retrieved (Fig. 2A). In contrast, most of the implants seeded with ALDH−/CD44−Lin− did not generate tumors. And, in the few instances (2 of 15 implants) that tumors were generated from these cells, they were structurally disorganized and had smaller tumor islands than xenografts generated from ALDH+/CD44+Lin− cells (Fig. 2A). These findings, along with the observation that tumor xenografts maintained a complex cellular composition and a proportion of ALDH+/CD44+ cells that was comparable with the primary tumor throughout 2 serial passages in vivo, indicated that ALDH+/CD44+Lin− cells exhibit features of multipotency. The epithelial origin of the tumor xenografts was confirmed by positive immunostaining for pancytokeratin (Fig. 2B). Analysis of the localization of the stem-like cells (ALDH-positive) within these xenografts revealed that the majority of these cells were found within 100 μm of blood vessels (Fig. 2C and D). This observation led us to a more in depth analysis of the localization of stem-like cells in primary tumors.

**Head and neck cancer stem-like cells exhibit perivascular localization**

In oral mucosa, the small subpopulation of ALDH-positive cells were found primarily in the basal layer of the squamous epithelium (Fig. 3A), the expected localization of stem cells in this tissue. In HNSCC, the ALDH-positive cells were seen in tumor islands, in close proximity to blood vessels (Fig. 3A). To assess the relative percentage of putative stem cells in oral mucosa and HNSCC, we prepared single cell suspensions and sorted them for ALDH and CD44. The proportion of ALDH+/CD44+ was lower (P = 0.004) in normal human oral mucosa (0.31 ± 0.11%) than in the HNSCC (2.62 ± 0.68%) (n = 3). Confocal microscopy and 3-D image reconstruction were used to evaluate the spatial relationship between ALDH-positive cells and blood vessels in 8 patients with HNSCC (Fig. 3B–D). An area with 100-μm radius around each blood vessel was selected as representative of the “perivascular” area, since this is the approximate area of diffusion of oxygen and nutrients around vessels (30). We observed that the majority of the CSC (i.e., approximately 80%) was found in the perivascular area in human HNSCC (Fig. 3D).

**Endothelial cell-derived growth factor milieu promotes proliferation, survival, and self-renewal of HNSC**

To understand whether endothelial cell-secreted factors have a direct functional effect on HNSC, we studied the effect of endothelial cell CM on proliferation, survival and self-renewal of ALDH+/CD44+ selected from a panel of established head and neck tumor cell lines in vitro. The proliferation of both, ALDH+/CD44+ and ALDH−/CD44− cells cultured in low attachment conditions was enhanced by exposure to endothelial cell CM (Supplementary Fig. S4A). The increase in cell numbers may also be attributed to an enhancement in survival mediated by the endothelial cell-derived factors (Supplementary Fig. S4B). In addition, we performed a series of supporting in vitro experiments using a panel of established HNSCC cell lines. The presence and proportion of putative CSC is depicted in Supplementary Figure S5. To evaluate the self-renewal potential of CSC, we plated them in agarose and observed the formation of colonies in a 3-D culture condition (Supplementary Fig. S6). We observed that the formation of sphere-like colonies developed from single cells (Fig. 4A and B), using a method inspired by the work on “mammospheres” (28). These colonies derived from head and neck tumor stem-like cells were named “orospheres.” The number of colonies generated from ALDH+/CD44+ cells was greater than ALDH−/CD44− cells (P < 0.001), using cells sorted from 3 established HNSCC cell lines, that is, UM-SCC-17A, UM-SCC-1, and UM-SCC-74A (Fig. 4A). To evaluate the behavior of these cells over time, the orospheres were dissociated and passed twice. Although the overall number of orospheres decreased over time, the ALDH+/CD44+ group persistently presented higher number of orospheres than the control group over 3 serial passages in vitro (Fig. 4B). Notably, ALDH+/CD44+ cells strongly express the marker of self-renewal Bmi-1 as compared with control ALDH−/CD44− cells (Fig. 4C). In the same experiment, we observed that endothelial-cell secreted factors enhances expression of Bmi-1 in ALDH+/CD44+ cells over time, indicating an inductive effect of these factors on the self-renewal properties of the CSC (Fig. 4C). To further understand the effect of endothelial cells on self-renewal and survival of CSC, we isolated ALDH+/CD44+Lin− cells from primary HNSCC and performed the orosphere assay with primary cells. A 3-fold increase in the number of orospheres was observed in the group treated with endothelial cell CM (P < 0.001) as compared with untreated controls (Fig. 5A). These results were verified in experiments performed with 6 additional head and neck tumor cell lines.
Notably, the inductive effect of endothelial cell-secreted factors on the number of orthospheres generated from ALDH<sup>+</sup>CD44<sup>+</sup>CD44<sup>-Lin/C0</sup> cells was maintained during 3 serial passages in vitro (Fig. 5C).

A critical question that remained unanswered was if the effects of endothelial cells on the survival and self-renewal of head and neck tumor stem cells was also observable in vivo (Fig. 5D).

**Selective ablation of blood vessels reduces the proportion of cancer stem cells**

A critical question that remained unanswered was if the effects of endothelial cells on the survival and self-renewal of head and neck tumor stem cells was also observable in vivo (Fig. 5D).

To address this question, we utilized 2 complementary approaches. First, we performed a serial dilution experiment with 1,000, 100, 10, or 1 ALDH<sup>+</sup>CD44<sup>+</sup>Lin<sup>-</sup> cells from a primary HNSCC seeded in the scaffolds with or without HDMEC. Coimplantation of endothelial cells and CSC resulted in larger tumors than implantation of CSC by themselves (Supplementary Fig. S7). Second, endothelial cells were stably transduced with a caspase-based artificial death switch (iCaspase-9) (Fig. 6A). This unique approach allows for selective elimination of endothelial cells transduced with iCaspase-9 on activation by the dimerizer drug AP20187 and ablation of tumor vasculature in vivo (27, 31). Here, we showed that treatment with AP20187 induces apoptosis of HDMEC–iCaspase-9, but not untransduced

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**Figure 2.** Head and neck cancer stem-like cells generate xenografts with histological features that closely resemble those of the original primary tumors. A, photomicrographs of a representative primary tumor (HN10), and respective primary and secondary xenograft originated by the transplantation of ALDH<sup>+</sup>CD44<sup>+</sup>Lin<sup>-</sup> cells (HE staining). Control tissues generated by the implantation of ALDH<sup>-</sup>CD44<sup>-</sup>Lin<sup>-</sup> cells. B, expression of pancytokeratin in the primary tumor, respective primary and secondary xenograft, and IgG control group. C, representative photomicrographs of ALDH1 immunostaining of primary tumors and respective xenografts. Arrows, ALDH1 positive cells. D, graph shows the percentage of ALDH1 positive cells found within a 100-μm radius of blood vessels in the primary tumors and xenografts generated by the transplantation of ALDH<sup>+</sup>CD44<sup>+</sup>Lin<sup>-</sup> cells.
cells (i.e., ALDH$^+$CD44$^+$ cells) (Fig. 6B). As expected, AP20187-induced apoptosis of the iCaspase-9–transduced endothelial cells resulted in a significant reduction in the microvessel density of tumors retrieved from mice injected with AP20187, as compared with vehicle-treated controls (Fig. 6C). Because of the short-term nature of the treatment with AP20187 (4 days), we have not observed a significant change in tumor size (data not shown). However, the fraction of ALDH$^+$CD44$^-$ cells within the xenograft tumors was significantly reduced when endothelial cells

Figure 3. Head and neck cancer stem-like cells are localized in close proximity to blood vessels. A, representative photomicrographs of tissue sections stained with HE or immunostained for ALDH1 in human HNSCC and control oral mucosa. Arrows, ALDH1 positive cells. B, confocal microscopy of human HNSCC immunostained for ALDH1 (green) and Factor VIII (red) for localization of blood vessels. The overlay image shows the perivascular localization of the ALDH-positive cells. C, 3-D reconstruction of the overlay image shown in B, depicting the spatial relationship between ALDH positive cells (green) and blood vessels (red). D, graph shows the percentage of ALDH$^+$ cells found within a 100-μm radius of a blood vessel. Analysis was performed in 6 random areas of 6 different tissue sections from each individual human HNSCC ($n = 8$).
were selectively ablated by the activation of iCaspase-9 as compared with vehicle-treated controls (Fig. 6D).

Discussion

The poor survival and high recurrence rates in patients with HNSCC demand a reassessment of the pathobiology of these cancers. Here, we showed that HNCSC reside in perivascular niches. Notably, we observed that endothelial cell-secreted factors have a major impact on the self-renewal and survival of CSC. These data suggest that therapeutic targeting of the tumor endothelium may reduce the rate of head and neck tumor recurrence and metastasis by decreasing the proportion of CSC.

Prince and colleagues reported that CD44+/CD44− cells sorted from UM-SCC-74A and grown in 3-D agarose matrices. The graph shows the quantification of the number of colonies arising from cancer stem-like cells (CSC; ALDH+/CD44+) or noncancer stem cells (NCSC; ALDH−/CD44−) sorted from 3 HNSCC cell lines (UM-SCC-17A, UM-SCC-1, UM-SCC-74A) and cultured in 3-D agarose matrices. B, representative photomicrographs of the number of “orospheres” from serial passage assays that evaluate self-renewal of CSC (ALDH+) compared with vehicle-treated controls (Fig. 6D). C, Western blot depicting the expression of Bmi-1 in CSC (ALDH+) and NCSC (ALDH−) cells treated with endothelial cell conditioned medium (EC CM).

Figure 4. ALDH and CD44 select for a subpopulation of cells that exhibit self-renewal properties in vitro. A, representative photomicrographs of colonies (orospheres) arising from ALDH+/CD44+ and ALDH−/CD44− cells sorted from UM-SCC-74A and grown in 3-D agarose matrices. The graph shows the quantification of the number of colonies arising from cancer stem-like cells (CSC; ALDH+/CD44+) or noncancer stem cells (NCSC; ALDH−/CD44−) sorted from 3 HNSCC cell lines (UM-SCC-17A, UM-SCC-1, UM-SCC-74A) and cultured in 3-D agarose matrices. B, representative photomicrographs of orospheres arising from ALDH+/CD44+ and ALDH−/CD44− cells sorted from UM-SCC-74A and grown in ultra-low attachment plates. The graph shows the number of orospheres arising from cancer stem-like cells (CSC; ALDH+/CD44+) or noncancer stem cells (NCSC; ALDH−/CD44−) cells in vitro. *P < 0.001. C, Western blot depicting the expression of Bmi-1 in CSC (ALDH+/CD44+) and NCSC (ALDH−/CD44−) cells treated with endothelial cell conditioned medium (EC CM).
in neural tumors (20, 35). Antiangiogenic agents (e.g., bevacizumab) have been shown to mediate a depletion in the CSC in models of gliomas and medulloblastomas. Here, we used a unique experimental approach to selectively eliminate tumor-associated endothelial cells and evaluate the effect on the stem cell compartment. Unlike previous experimental strategies that were based on antiangiogenic drugs, the approach here eliminates the risk of a direct effect of the drug on the viability or stemness of the tumor stem cells. We observed that selective ablation of tumor-associated blood vessels is sufficient to decrease the proportion of head and neck tumor stem cells within 4 days, whereas no changes were observed in tumor volume in the same time period.

Our work demonstrates that endothelial cells initiate signaling events that enhance the survival and self-renewal of stem cells in head and neck tumors. In addition, the data presented here supports the concept that head and neck cancer indeed follows the CSC hypothesis, since implantation of few cells consistently gives rise to tumors that can be serially passaged in vivo. Collectively, these data suggest that therapeutic strategies that include antiangiogenic agents might have the benefit of reducing the proportion of CSC in head and neck tumors. These results might translate into

Figure 5. Endothelial cell-derived factors promote proliferation and self-renewal of head and neck cancer stem-like cells. A, photomicrographs of representative colonies arising from ALDH$^+$CD44$^+$Lin$^-$ cells sorted from a human HNSCC and grown in 3-D agarose matrices. Cells were treated with endothelial cell conditioned medium (CM) or unconditioned control medium (CT) for 1 week. *, $P < 0.001$. B, time course experiment depicting the number of orospheres arising from CSC (ALDH$^+$CD44$^+$) and NCSC (ALDH$^-$CD44$^-$) cells treated or not with endothelial cell CM over a period of 4 weeks. C, photomicrographs of representative colonies arising from ALDH$^+$CD44$^+$Lin$^-$ cells sorted from UM-SCC-74A cells and cultured in ultra-low attachment plates. Cells were treated with endothelial cell CM or unconditioned CT. The graph shows primary, secondary, and tertiary orospheres arising from ALDH$^+$CD44$^+$ treated with endothelial cell CM or CT. *, $P < 0.05$. 

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lower recurrence rates and better survival of head and neck cancer patients.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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