Original Research—Head and Neck Surgery

Effect of Adipose-Derived Stem Cells on Head and Neck Squamous Cell Carcinoma

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Abstract

Objective. Patients with head and neck squamous cell carcinoma (HNSCC) have significant wound-healing difficulties. While adipose-derived stem cells (ASCs) facilitate wound healing, ASCs may accelerate recurrence when applied to a cancer field. This study evaluates the impact of ASCs on HNSCC cell lines in vitro and in vivo.

Study Design. In vitro experiments using HNSCC cell lines and in vivo mouse experiments.

Setting. Basic science laboratory.

Subjects and Methods. Impact of ASCs on in vitro proliferation, survival, and migration was assessed using 8 HNSCC cell lines. One cell line was used in a mouse orthotopic xenograft model to evaluate in vivo tumor growth in the presence and absence of ASCs.

Results. Addition of ASCs did not increase the number of HNSCC cells. In clonogenic assays to assess cell survival, addition of ASCs increased colony formation only in SCC9 cells (maximal effect 2.3-fold, \( P \leq .02 \)) but not in other HNSCC cell lines. In scratch assays to assess migration, fluorescently tagged ASCs did not migrate appreciably and did not increase the rate of wound closure in HNSCC cell lines. Addition of ASCs to HNSCC xenografts did not increase tumor growth.

Conclusion. Using multiple in vitro and in vivo approaches, ASCs did not significantly stimulate HNSCC cell proliferation or migration and increased survival in only a single cell line. These findings preliminarily suggest that the use of ASCs may be safe in the setting of HNSCC but that further investigation on the therapeutic use of ASCs in the setting of HNSCC is needed.

Keywords

adipose stem cells, wound healing, head and neck reconstruction, radiation injury, head and neck cancer, squamous cell carcinoma
undergo self-renewal while maintaining the ability to differentiate into multiple cell lineages and tissue phenotypes. ASCs are more easily obtained than bone marrow stem cells but exhibit similar wound-healing properties, which are thought to be due to promotion of angiogenesis, secretion of growth factors and cytokines, and/or differentiation into multiple cell types. Recent studies have demonstrated the ability of ASCs to improve wound healing in flap models, ischemic models, radiation injury models, and diabetes, suggesting a potential role in reconstruction and wound healing for patients with HNSCC.

Despite the apparent benefits, surgical oncologists have concerns that, given their growth-promoting properties, ASCs may cause or accelerate recurrence; there has been limited research on the effect of ASCs on HNSCC to support or refute this concern. The purpose of this study is to preliminarily identify and describe the response of HNSCC cell lines to the introduction of ASCs in vitro and in vivo, an initial investigation paramount to advancing the clinical application of ASCs for reconstruction and wound healing in patients with HNSCC.

Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM)/F-12 media with HEPES and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, California). Hydrocortisone was purchased from Sigma-Aldrich (St Louis, Missouri).

Tissue Culture

SCC25, SCC9, Cal27, FaDu, and Rat1 cells were obtained from ATCC (Manassas, Virginia). SCC61, UNC7, and UNC10 cells were kindly provided by Dr Wendell Yarbrough (Yale University, New Haven, Connecticut). OSC19 cells were kindly provided by Dr Jeffrey Myers (The University of Texas MD Anderson Cancer Center, Houston, Texas). Cell line identities were confirmed by DNA fingerprinting (University of Arizona). All cells were grown in DMEM/F-12 media supplemented with 5% FBS and 400 ng/mL hydrocortisone and maintained in a 37°C humidified incubator with 5% CO2. All cell lines were routinely tested and found to be free of mycoplasma contamination using MycoAlert (Lonza, Allendale, New Jersey).

Acquisition of ASCs

ASCs used were derived from remnant human adipose tissue isolated from elective procedures performed on healthy patients by the University of Virginia Plastic Surgery service under an institutional review board–approved protocol. Harvested adipose tissue was minced, homogenized, and washed followed by enzymatic digestion. The resultant solution was centrifuged; the pellet containing the stromal vascular fraction (SVF) was transferred to culture bottles and incubated. The cells proliferated, becoming undifferentiated mesenchymal cells. For certain studies, ASCs were treated with Vybrant Dil Cell-Labeling Solution (ThermoFisher Scientific, Waltham, Massachusetts), a lipophilic tracer delivery solution that can be added directly to normal culture media to uniformly label adherent cells with a red fluorescent marker. ASCs were labeled to ascertain their location and relationship to cancer cells in vitro.

Luciferase Transfection

FaDu, Cal27, and Rat1 cell lines were transfected with the complementary DNA (cDNA) encoding firefly luciferase (pGL4.51 Luciferase; Promega, Madison, Wisconsin) and selected with G418 to produce stable FaDu-Luc, Cal27-Luc, and Rat1-Luc cells. OSC19 cells were luciferase expressing when obtained.

Bioluminescent Proliferation Assay

Cell lines were seeded in a white, opaque 96-well plate and grown in DMEM/F12 medium supplemented with 0.5% FBS. Rows containing 1250, 2500, and 5000 cells/well were used to calibrate sensitivity of the plate reader and to confirm linear analytical response of the assay. Experimental wells were seeded with 2500 HNSCC cells. After 24 hours, the experimental wells were seeded with 0, 500, 1000, or 2000 ASCs and incubated at 37°C for 72 hours. Plates were treated with the Luciferase Assay System according to the manufacturer’s protocol for plate reading luminometers (Promega). Luminescence was read using the Synergy 2 Multi-Mode Reader (BioTek, Winooski, Vermont) and plotted in relative luminescence units (RLUs), a standardization of the luminescent product based on the control (cells cultured without ASCs) luminescence. ASCs were also plated alone to confirm they did not produce noise that would contribute to luminescence signal from transfected cells. A RLU was calculated for the addition of 500, 1000, and 2000 ASCs in comparison to the control (no ASCs) for each cancer cell line. Each experimental condition was performed in triplicate.

Clonogenic Survival Assay

Optimal dilution was determined empirically for each cell line such that the maximal number of colonies was approximately 100. Cells were plated in 6-well plates at the appropriate density with or without ASCs. The plates were then incubated for 9 days, during which time the media were changed twice. After 9 days, the plates were stained with crystal violet for 20 minutes and then washed. The number of colonies was manually counted.

To determine if ASCs form colonies, they were labeled with DiI (red fluorescent membrane tag), plated at a low density without or with SCC9 cells, and observed for 2 weeks using fluorescence microscopy. ASCs did not form colonies either alone or in combination with SCC9 cells. When cocultured with HNSCC cells, ASCs were noted to be distributed around and within colonies of HNSCC cells (data not shown).

Scratch Migration Assay

Before plating, 6-well plates were marked with a straight line across all the wells. Scratches were subsequently made
perpendicular to the mark. The site of intersection was used to consistently evaluate the same location under the microscope. HNSCC cell lines were plated without and with 10,000 ASCs fluorescently tagged with Dil and allowed to grow for 48 hours to achieve confluence. The culture medium was changed to 0.5% FBS 12 hours prior to placement of scratches in the well. A 200-μL pipette tip was used to create 3 scratches per well that intersected the previously placed mark. After the scratches were made, each well was gently rinsed with phosphate-buffered saline (PBS). Images were obtained at 0, 8, and 24 hours using the EVOS FL Auto Cell Imaging System (Invitrogen) that detects nonfluorescent squamous cells by phase contrast microscopy and fluorescent ASCs using simultaneous fluorescence microscopy (using the RFP crystal). The remaining scratch area was calculated using National Institutes of Health (NIH) ImageJ software and normalized to the area of the same scratch at time 0 hours. For each cell line, 3 experiments were performed in triplicate.

Orthotopic tumor model. Tongue tumors were developed in female nude mice by submucosal injection of OSC19 cells as previously described. A group of 10 mice was divided into 2 groups of 5: group 1 was injected with 30,000 OSC19 cells, and group 2 was injected with 30,000 OSC19 cells, followed by an intratumoral injection of 30,000 ASCs 7 days later. Mice were imaged by bioluminescence imaging (BLI) every 7 days, beginning on the day of injection, for 3 weeks using the IVIS-200 Imaging System (Xenogen Corporation, Berkeley, California).

Bioluminescence imaging analysis. The luminescent area of the xenograft tumor was defined as the region of interest (ROI), and the total signal in the ROI (photons/s/cm²/sr) was quantified using Living Image 3D software version 1 (Xenogen Corporation). The ROI was applied to all images of the xenograft tumor to identify using Living Image 3D software version 1 (Xenogen Corporation). The ROI was applied to all images of the same sequence of each tumor. The total signal intensity (photons/s/cm²/sr) was plotted against time to measure tumor growth.

Statistical analysis. Statistical analysis was performed using Student 2-tailed t tests. Findings were considered statistically significant when P < .05.

**Results**

**ASCs Do Not Stimulate Proliferation of HNSCC Cell Lines In Vitro**

As shown in Figure 1A-C, addition of ASCs to luciferase-expressing Cal27, OSC19, and FaDu cells did not cause an increase in RLU, a surrogate for cell number. In the case of Cal27 cells, there was approximately a 70% decrease in RLU with addition of as few as 500 ASCs (P = .013), while OSC19 showed a 50% reduction only when 2000 ASCs were added (P = .028). Addition of up to 2000 ASCs had no impact on FaDu cells. Addition of 2000 ASCs to Rat1-Luc fibroblasts resulted in a 2.1-fold increase in cell number (P = .03, Figure 1D) demonstrating the viability and pro-proliferative potential of the ASCs.

**ASCs Do Not Promote HNSCC Migration In Vitro**

Having observed no pro-proliferative effect of ASCs on HNSCC cell lines, a clonogenic assay was used to determine the effect of ASCs on HNSCC cell survival. When tested alone, ASCs did not demonstrate colony formation; they instead formed a thin layer of dispersed cells across the plate. When plated with cancer cells, ASCs were noted to be located in and around colonies but not form colonies themselves (data not shown).

Eight HNSCC cell lines were tested using the clonogenic survival assay as shown in Figure 2A-H. In each case, 3 different HNSCC cell dilutions were plated (only data obtained with 250 HNSCC cells are shown in Figure 2) and combined with 4 different quantities of ASCs. Of this panel of cell lines, only SCC9 showed improved survival with the addition ASCs: 2000 and 5000 ASCs increased the number of SCC9 colonies 2.0- and 2.3-fold, respectively (P < .02, Figure 2F). To confirm prosurvival activity of the ASCs, Rat1 fibroblast cells were also tested and demonstrated a significant 1.7- and 1.8-fold increase in survival with addition of 2000 and 5000 ASCs, respectively (P < .02).

**ASCs Do Not Accelerate HNSCC Migration In Vitro**

A scratch assay was used to determine the effect of ASCs on HNSCC cell migration in vitro. To determine which cell type is responsible for wound closure, previously Dil-labeled ASCs and HNSCC cells were imaged with simultaneous phase contrast and fluorescence microscopy. As shown in Figure 3A,B, ASCs did not contribute to wound closure directly. Also of note in Figure 3, scratch wounds become very irregular as they closed; thus, wound area rather than gap width was calculated to evaluate wound closure and compared to the initial scratch wound.

Seven HNSCC cell lines and Rat1 fibroblasts were studied via scratch assay, and all showed progressive reduction in wound area at 8 hours and 24 hours (Figure 4A,B). SCC25 and Rat1 cells had completely closed the wound at 24 hours. In all cases, including Rat1 fibroblasts, addition of ASCs had no statistically significant impact on the amount of wound closure (Figure 4A,B), indicating that ASCs did not alter the rate of migration.

**ASCs Do Not Promote HNSCC Tumor Growth In Vivo**

To assess the effects of ASCs on tumor growth in vivo, luciferase-expressing OSC19 cells were injected into the tongues of nude mice alone or with ASCs. The mice were followed using BLI for 3 weeks. In the initial in vivo experiment, simultaneous injection of ASCs resulted in poor tumor take and little or no tumor growth despite normal growth of control tumors (data not shown). This was thought to be due to the simultaneous injection technique and not a biologic effect of the ASCs on the OSC19 cells. In the subsequent in vivo experiment, OSC19 cells were injected in all animals, and tumors were allowed to grow...
for 1 week. Half of the established tongue tumors were then injected with ASCs on day 7. As shown in Figure 5, delayed injection of ASCs did not affect the overall tumor growth pattern. Thus, no augmentation of tumor growth was noted in vivo when ASCs were injected into orthotopic xenograft tumors.

**Discussion**

Several animal studies have investigated the healing benefits of ASCs. In mice, fat grafting alleviates radiation skin damage by attenuating inflammation and slowing fibrosis. In rats with radiation ulcers, ASCs promote neovascularization and accelerate wound healing. Random pattern skin flaps exhibit increased capillary density after ASC application, and flap perfusion and survival are improved after addition of ASCs, which enhance expression of proangiogenic and proinflammatory genes. There have also been human studies using ASCs. In a study by Rigotti et al, ASCs were injected into patients with severe radiation wounds. The target tissue demonstrated progressive regeneration, and all patients had a systematic improvement or remission of their symptoms. In addition to improving skin and soft tissue wound-healing capabilities, ASCs have shown potential utility in improving vocal fold wound healing and regeneration of radiation-induced salivary gland damage.

Despite the vast potential benefit of ASCs, it is prudent to consider the potential risks. Patients treated for HNSCC may have residual microscopic disease, and the impact of potently pro-proliferative and proangiogenic ASCs is unknown. Studies on the effect of therapeutically delivered ASCs in the setting of cancer are limited. Most published studies relate to breast cancer due to greater concern of the increased presence of ASCs in the tumor environment of fatty breast tissue. Breast tissue–resident stem cells promote breast cancer growth and metastasis when added to a murine breast cancer cell line. Martin-Padura et al demonstrated that the adipose tissue in lipotransfer procedures is rich in CD34+ progenitors that contribute to tumor vascularization, growth, and metastases in several orthotopic models of human breast cancer. Perrot et al describe a rare case of local recurrence of osteosarcoma 13 years after the initial resection but only 18 months after a cosmetic lipofilling procedure. The recurrence occurred at the exact
site of the autologous fat grafts. Therefore, while ASCs have the potential to promote wound healing and functional regeneration, it is necessary to demonstrate that they do not promote cancer progression prior to their clinical application in patients with HNSCC. The only published study to date investigating the impact of ASCs on HNSCC by Rowan

**Figure 2.** Survival effect of adipose-derived stem cells (ASCs) on head and neck squamous cell carcinoma colony formation. Graphs represent mean colonies formed ± standard error of the mean following a seeding density of 250 cancer cells. Rat1 cells were used as positive control. *P < .02, 2-tailed Student t test.

**Figure 3.** Migration behavior of head and neck squamous cell carcinoma cells co-cultured with adipose-derived stem cells. Images of a scratch assay of SCC9 cells plated with fluorescently tagged at 0 hours (A) and 24 hours (B).
et al\textsuperscript{17} reported that human ASCs did not affect growth of human HNSCC cells or tumor xenografts but stimulated migration and early micrometastasis to mouse brain. The present study corroborates and expands upon the findings of Rowan et al.\textsuperscript{17} Multiple HNSCC cell lines were tested for impact of ASCs on in vitro proliferation, survival, and migration. Contrary to the findings of Rowan et al,\textsuperscript{17} ASCs did not promote migration of HNSCC cells in the present study. Similarly, ASCs did not promote survival in 7 of 8 HNSCC cell lines and did not stimulate proliferation in 3 HNSCC cell lines. As a positive control, Rat1 fibroblasts showed statistically significant increases in proliferation and survival upon the addition of ASCs. The fact that only one of several HNSCC cell lines exhibited an enhanced survival in response to ASCs generates some optimism that ASCs may have little or no impact on HNSCC tumor growth, but this study only begins to assess this very heterogeneous group of tumors. The single prosurvival response suggests that a subset of HNSCC tumors may be ASC responsive, and thus much more study is warranted to better understand the mechanism of ASC-HNSCC interactions and the unique characteristics that could predispose tumor cells to a growth or survival response to ASCs. While the present study did not find increased tumor growth upon the addition of ASCs to HNSCC tumors in vivo, only a single cell line was studied. This cell line may not be widely representative of HNSCC cells, and the in vivo model used may not effectively replicate the tumor microenvironment in patients. Clinically, ASCs would likely be used in the context of poor wound healing after treatment, in which case we would expect few or no viable tumor cells to be present in most cases. In this setting, the ratio of ASCs to tumor cells and the tissue microenvironment would be extremely different from the in vivo model studied.

The present study provides initial evidence to suggest that ASCs may be safe for therapeutic use in at least some patients with HNSCC. It represents only a preliminary assessment, and future studies should assess the impact of ASCs on a broader group of HNSCCs, possibly via use of ex vivo culture, and also the mechanisms of ASC-stimulated tumor survival and growth. While this study evaluated a limited number of cell lines, it suggests that at least a large subset of HNSCC tumors will not respond to ASCs. There remains a risk that some tumors could exhibit enhanced cell survival (as demonstrated in Figure 2) or even growth in the presence of ASCs, and this needs to be further explored. In addition, ASCs from nonhealthy patients should be evaluated to see if there are patient-to-patient differences in the responses induced by the addition of ASCs.

**Conclusion**

In vitro, ASCs did not significantly stimulate proliferation or migration of multiple HNSCC cell lines and increased clonogenic survival in only 1 of 8 HNSCC cell lines. Simultaneous or delayed addition of ASCs to an orthotopic murine xenograft model did not accelerate tumor growth in vivo. These findings provide preliminary evidence that ASCs may be safe in the setting of HNSCC, with the caveat that a subset of tumors may be responsive. Further investigation into the therapeutic
use of ASCs in the setting of HNSCC is necessary and warranted.

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Author Contributions

Deep Danan, study conception/design, data acquisition/analysis, data interpretation, drafting manuscript, final approval, accountable for data; Christine E. Lehman, data acquisition/analysis, data interpretation, drafting manuscript, final approval, accountable for data; Rolando E. Mendez, data acquisition/analysis, data interpretation, drafting manuscript, final approval, accountable for data; Brian Langford, data acquisition/analysis, revision of manuscript, final approval, accountable for data; Paul D. Koors, data acquisition/analysis, revision of manuscript, final approval, accountable for data; Michael I. Dougherty, data acquisition/analysis, data interpretation, revision of manuscript, final approval, accountable for data; Shayn M. Peirce, study conception/design, revision of manuscript, final approval, accountable for data; Daniel G. Gioeli, data interpretation, revision of manuscript, final approval, accountable for data; Mark J. Jameson, study conception/design, revision of manuscript, final approval, accountable for data.

Disclosures

Competing interests: Mark J. Jameson is a member of the advisory panel for AstraZeneca.

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References