The Effect of Mesenchymal Stromal Cell–Hyaluronic Acid Hydrogel Constructs on Immunophenotype of Macrophages

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During the past several years, multipotent mesenchymal stromal cells (MSCs) have rapidly moved from *in vitro* and animal studies into clinical trials as a therapeutic modality potentially applicable to a wide range of disorders. It has been proposed that *ex vivo* culture-expanded MSCs exert their tissue regeneration potential through their immunomodulatory and anti-inflammatory properties, and paracrine effects more than their ability to differentiate into multiple tissue lineages. Since extracellular matrix (ECM) deposition and tissue support is also one of many physiological roles of MSCs, there is increasing interest in their potential use for tissue engineering, particularly in combination with ECM-based scaffolds such as hyaluronic acid (HA). We investigated the effect of MSCs on immunophenotype of macrophages in the presence of an HA–hydrogel scaffold using a unique 3D coculture system. MSCs were encapsulated in the hydrogel and peripheral blood CD14⁺ monocyte-derived macrophages plated in direct contact with the MSC–gel construct. To determine the immunophenotype of macrophages, we looked at the expression of cell surface markers CD14, CD16, CD206, and human leukocyte antigen (HLA)–DR by flow cytometry. MSCs and macrophages cultured on the HA–hydrogel remained viable and were able to be recovered from the construct. There was a significant difference in the immunophenotype observed between monocyte-derived macrophages cultured on the HA scaffold compared to tissue culture polystyrene. Macrophages cultured on gels with MSCs expressed lower CD16 and HLA–DR with higher expression of CD206, indicating the least inflammatory profile overall, compatible with the immunophenotype of alternatively activated macrophages. Development of macrophages, with this immunophenotype, upon interaction with the MSC–hydrogel constructs may play a potentially significant role in tissue repair when using a cellular–biomaterial therapeutic approach.

Introduction

CHEMICALLY MODIFIED HYALURONIC ACID (HA) hydrogels have been used in a variety of preclinical studies and clinical applications for soft tissue regeneration. Most notable are commercially available HA-based materials for injectable soft tissue fillers, coating materials on implantable devices, or dermal replacement grafts.¹ Hyaluronan is a vital element of the extracellular matrix (ECM), providing tissues with an optimal environment for cell attachment, migration, and growth.² In addition, HA is an important player in the inflammatory cascade, not only recruiting immune cells to the site of wounded tissue but contributing to granulation tissue formation as well.² While there has been an increase in the utilization and indications of natural and synthetic materials in regenerative medicine, the dual role of HA in tissue healing, that is, modulation of inflammatory cascade and

reparative properties, make it a potentially advantageous biomaterial for use in combination with emerging cell-based therapies.

There is an increasing interest in using multipotent mesenchymal stromal/stem cells (MSCs) derived from bone marrow (BM) or adipose tissue (AT) as a novel approach to tissue regeneration and healing.^{3–5} These progenitor cells isolated from different tissue sources all have the capability to differentiate into multiple cell lineages (such as bone, fat, and cartilage), and have specific cell surface marker characteristics,^{6–8} though there is evidence to suggest that these cells can have phenotypical differences based on their tissue source of origin.⁹ Most recently, unique immunomodulatory properties of MSCs, such as their potential use across HLA barriers, have been an area of active investigation as such properties make them of much more value in regenerative medicine.^{10–13} Although immunomodulatory properties of

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MSCs have been extensively studied in the context of their interactions with adaptive immune cells, such as T and B lymphocytes,^{14,15} their potential interactions with innate immune cells, such as macrophages, is not as well understood. Our group has recently shown that in the presence of MSCs *in vitro*, human macrophages not only express a higher level of cell surface marker CD206, but also assume other characteristics of anti-inflammatory type-II macrophages.¹⁶ This potential interaction is of particular significance given the major role of macrophages in regulating wound healing, inflammation, and soft tissue regeneration after a variety of insults.^{17,18}

Since hydrogels can be utilized alone or in combination with MSCs, our group set out to compare the effect of MSCs derived from different tissue sources on monocyte-derived macrophages in the presence of a HA-based hydrogel scaffold, Carbylan-GSX (Glycosan). In addition to BM- and AT-MSCs, we selected a well-established vocal fold (VF) fibroblast model.¹⁹ Our group recently characterized these latter cells as VF-MSCs⁷ based on the consensus criteria developed to define MSC populations.²⁰ Previous animal work with Carbylan GSX has shown that this material is biocompatible and nontoxic when cultured with VF-MSCs; furthermore, it enhances overall healing and tissue regeneration in an animal model of inflammation.^{2,21,22} Here, we developed a unique three-dimensional (3D) coculture system with MSCs encapsulated in the HA-based hydrogel and macrophages derived from peripheral blood CD14⁺ monocytes plated in direct contact with the MSC-gel construct to investigate the cell surface marker expression of monocyte-derived macrophages vital to tissue healing and inflammation (Fig. 1). We hypothesized that monocyte-derived macrophages differentiated in the presence of this MSC-hydrogel construct will exhibit an anti-inflammatory immunophenotype.

Materials and Methods

Human MSC isolation

Human MSCs were derived from BM, AT, and VF of healthy donors based on protocols approved by the University of Wisconsin Health Sciences Institutional Review Board as previously described.^{7,23} BM- and AT-MSCs were cultured in a 37°C incubator with 5% CO₂–95% humidified atmosphere with standard media (alpha minimum essential media supplemented with 10% fetal bovine serum [FBS-Hyclone, Logan, UT], 1×nonessential amino acids [NEAA, Sigma Aldrich], and 4mM L-glutamine [Sigma Inc.], 100 U/mL penicillin, and 0.01 mg/mL streptomycin sulfate [Sigma Inc.]). VF-MSCs were cultured in Dulbecco's modified essential medium with 10% FBS, 100 U/mL penicillin, 0.01 mg/mL streptomycin sulfate, and 1×NEAA (all from Sigma Inc.). The medium was changed every 3 days and the cells were expanded until passages four to seven, at which time they were used for this study.

Human monocyte isolation

Buffy coat samples were obtained from normal healthy donors (Interstate Blood Bank) and mononuclear cells isolated by density grade centrifugation. Mononuclear cells were then incubated with anti-human CD14 microbeads (Miltenyi Biotec) for 15 min at 4°C and washed to remove unbound antibody. A pure population of CD14⁺ cells was collected using an AutoMACS Pro Separator (Miltenyi Biotec) according to manufacturer's instructions. This method has previously been shown to yield >95% cell purity via flow cytometry in our laboratory.¹⁶ The cells were stored at –80°C until use for this study.

FIG. 2. Representative photomicrographs of monocyte-derived macrophages cultured on TCPS (**top row, A–D**) or Carbylan-GSX (**bottom row, E–H**) taken on day 7. CD14⁺ cells were cultured in 2D coculture conditions on TCPS alone (**A**) or with bone marrow (BM)-derived MSCs (**B**), adipose tissue (AT)-derived MSCs (**C**), or vocal fold (VF) MSCs (**D**). Alternatively, CD14⁺ cells were cultured on top of Carbylan-GSX 3D hydrogel alone (**E**) or with encapsulated BM-MSCs (**F**), AT-MSCs (**G**), or VF-MSCs (**H**). All images taken at 10 $\times$ magnification.

Hydrogel preparation

A chemically modified hyaluronan–gelatin hydrogel (Carbylan-GSX) has been previously developed in collaboration with the Center for Therapeutic Biomaterials at the University of Utah.^{24–26} Briefly, this was prepared consisting of 8.2% polyethylene glycol diacrylate (MW 3.4 kDa), 1.4% crosslinked thiol-modified hyaluronan 3,3'-dithiolbis (propanoic hydrazide) (HA-DTPH), and 1% thiol-modified gelatin-DTPH.

Cell culture

Tissue culture polystyrene (TCPS) six-well plates were used for the coculture experiments. For 2D culture conditions, BM-, AT-, or VF-MSCs (2×10^5 cells) were added to the six-well plate (Fig. 1A). For 3D culture conditions, BM-, AT-, and VF-MSCs were mixed into the Carbylan-GSX at a final concentration of 2×10^6 cells/mL of gel, and 500 μ L of the cell seeded gel was cast using a 0.4 μ m pore sized transwell (Corning) in the six-well plate (Fig. 1B). Carbylan-GSX was cast without cells as a control culture condition. The composite gel was incubated for 10 min at 37°C with 5% CO₂–95% humidified atmosphere to allow for polymerization. Within 2 h of plating the MSCs in 2D and 3D conditions, isolated CD14⁺ cells (1×10^6 cells) were thawed, washed with phosphate-buffered saline (PBS), and added to the surface of the hydrogel or TCPS. Cells were cultured in phenol red free RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 1% sodium pyruvate, and 1% NEAA for 7 days (Fig. 2). On day 4, the medium was removed and centrifuged at 1200 RPM for 10 min to collect any nonadherent CD14⁺ cells. The cells were suspended in fresh media and added back to the appropriate well. The plates were kept in a 37°C incubator with 5% CO₂–95% humidified atmosphere.

Flow cytometry

After a 7-day culture, media including nonadherent cells were aspirated from each well. The adherent macrophages

were harvested from the polystyrene surface using a cell scraper. For all of the hydrogel conditions, three incubations with 500 μ L ethylenediaminetetraacetic acid–trypsin for 2 min each were performed with fresh media added in between incubations. The resulting cell suspensions were incubated with 10 μ L of Fc Receptor Blocking Agent (Milenyi Biotech) in the dark for 15 min at 4°C. Surface antibodies were then added and incubated for 30 min at 4°C in the dark. Antibodies used included anti-CD14 Alexa 488 (A488), anti-CD14 fluorescein isothiocyanate, anti-CD16 phycoerythrin, anti-CD206 allophycocyanin, and anti-human leukocyte antigen (HLA)-DR peridinin chlorophyll protein-Cy5.5. Propidium iodide (PI) was used to measure viability of a sampling of the CD14 cells. Isotype-specific antibodies were used as controls. All antibodies were obtained from BD Pharmingen. The cells were centrifuged at 1200 RPM for 10 min in washing buffer (2% FBS in PBS) and fixed in 4% paraformaldehyde in PBS. Cell surface marker staining data were acquired using an Accuri C6 flow cytometer (Accuri Cytometers, Inc.) within 24 h of staining. CFlow software (Accuri Cytometers, Inc.) was used to collect and analyze the data. Forward scatter and side scatter parameters were used to gate viable cells (Fig. 3), and CD14 expression was used to identify the macrophage population. The percentages of cells within each gate were determined by isotype-matched controls (Fig. 3).

Statistical analysis

Surface marker expression data were obtained in duplicate for monocyte-derived macrophages from three different healthy donors. Each donor was cocultured with two different healthy MSC donors per tissue source. The data are expressed as a percentage of surface marker expression ($\pm$ SD). We were interested in comparing the proportion of positive cell types observed under 8 different treatment conditions. Since the same three sets of cells were used for each treatment condition, we used a split-plot analysis of variance (ANOVA) with Fisher's protected least significance

FIG. 3. Gating strategy and isotype control panels for CD14⁺ cells cultured on the surface of (A) TCPS dishes or (B) Carbylan-GSX hydrogel. Color images available online at www.liebertonline.com/tea

difference tests to look for differences between treatment groups. Before analysis, the data were transformed using an arcsine square root transformation to better meet the assumptions of ANOVA. An overall F-test significance level of 0.10 was used and otherwise, p -values <0.05 were considered as significant. All analyses were performed using SAS statistical software version 9.1 (SAS Institute, Inc.). Statistical significance of $p < 0.05$ is indicated by an (*) in the figures.

Results

Coculture system

Monocyte-derived macrophages were cocultured on the hydrogel/MSC culture system, which allowed for direct contact between macrophages and hydrogel. Additionally, monocyte-derived macrophages cultured in 2D on TCPS with or without MSCs were included as control (Fig. 1). Figure 2 shows representative photographs ($10\times$) for each of the culture conditions. We found that macrophages adhere to Carbylan-GSX with and without encapsulated MSCs. Cells could be removed from the hydrogel with a yield of $\sim 35\%$ ($2.5\text{--}6 \times 10^5$ cells compared to 1×10^6 cells originally plated on day 0). This number varied in the 2D conditions and those with encapsulated MSCs, as there were MSCs that were removed during the trypsinization process seen in our flow cytometry. Qualitatively, we found that the 2D coculture with macrophages and MSCs had greater nonadherent macrophages at the end of the seven day culture period compared to the 3D hydrogel cultures. In particular, plates containing VF fibroblasts had too few macrophages to provide meaningful analysis (Fig. 2D); therefore, the 2D MSC-TCPS analysis was performed using the BM and AT derived MSCs coculture data.

Cell viability

As a measure of macrophage viability after the 7-day coculture period with MSCs with or without an HA-based scaffold, PI was added to a sampling of cells from each

condition and gated in a manner similar to that shown in Figure 3. Cell viability was determined via total CD14⁺ cell number and percentage of PI staining. At the end of the 7-day coculture period, the viability of CD14⁺ cells removed from the culture conditions ranged from 95.6% to 99.2% with no difference among the groups (Fig. 4).

Immunophenotype of monocyte-derived macrophages

We have previously shown that the MSCs used in this study do not express hematopoietic markers such as CD14 and CD34.⁷ Figure 5 shows representative fluorescence-activated cell sorting (FACS) panels for the surface markers of interest, CD14, CD16, CD206, and HLA-DR, in each of the cultures (Fig. 5A–H). The left panel in each group shows CD14 plotted against CD206, whereas the right panel shows HLA-DR plotted against CD16. A composite of these data is shown in Figure 6. Overall, macrophages cocultured in 2D (TCPS) with MSCs expressed significantly higher CD16 and lower HLA-DR than those cocultured on HA-hydrogels containing MSCs in a 3D scaffold. Immunophenotype of macrophages was different among the four different culture conditions, indicating an effect of both the synthetic ECM (sECM) scaffold as well as MSC influence. As shown in Figure 7, the phenotype of CD14⁺ macrophages in the coculture groups includes low CD16, high CD206, and low HLA-DR expression compared to controls.

Expression of CD16 is lower on cells cocultured on MSC-hydrogel constructs and hydrogel alone

After 7 days of culture on TCPS, generated macrophages demonstrated a consistently high level of CD16 expression. The rate of expression was increased in coculture conditions including BM- and AT-MSCs on TCPS compared to macrophages alone on TCPS ($72\% \pm 21.6\%$ vs. $77\% \pm 18\%$ vs. $64\% \pm 22\%$, respectively). When we compared the expression of CD16 on macrophages cultured on Carbylan-GSX to TCPS, we found significantly lower event rates in the former ($35.5\% \pm 17.4\%$ vs. $64\% \pm 22\%$, respectively, $p < 0.001$). Finally,

FIG. 4. Viability of CD14+ monocyte-derived macrophages cocultured with MSCs on tissue culture polystyrene or hydrogel. Data are shown as mean expression $\pm$ SD. There was no statistical difference between the groups.

there was no difference observed in the rate of expression of CD16 among the hydrogel alone or any of the MSC-encapsulated groups ($35.5\% \pm 17.4\%$ hydrogel control vs. $35.2\% \pm 18.8\%$ hydrogel + BM-MSCs vs. $25.0\% \pm 20.9\%$ hydrogel + AT-MSCs vs. $27.6\% \pm 22.1\%$ hydrogel + VF-MSCs).

Expression of CD206 is elevated in all treatment groups

The expression of CD206 has been utilized as a marker of alternatively activated or anti-inflammatory macrophages.

FIG. 5. Surface marker expression of CD14+ monocyte-derived macrophages cocultured with MSCs on polystyrene or hydrogel and control macrophages. Cells were stained for CD14 and CD206; further, expression of CD16 and HLA-DR was analyzed for each condition. (A) TCPS control; (B) Carbylan-GSX control; (C) 2D TCPS with BM-MSCs; (D) 3D Carbylan-GSX with BM-MSCs; (E) 3D Carbylan-GSX with AT-MSCs; (F) 3D Carbylan-GSX with VF-MSCs (vocal fold fibroblast). Color images available online at www.liebertonline.com/tea

FIG. 6. Comparison of expression of CD16 and HLA-DR cell surface markers on macrophages cocultured in direct contact with MSCs in 2D TCPS culture or 3D encapsulated hydrogel culture. Controls include cultures without MSCs. Results are shown comparing mean event rate or count of CD16 and HLA-DR expression. Color images available online at www.liebertonline.com/tea

FIG. 7. Expression of CD16, CD206, and HLA-DR on CD14⁺ monocyte-derived macrophages (A) cultured on TCPS compared to Carbylan-GSX. (B) Expression of CD16, CD206, and HLA-DR on CD14⁺ monocyte-derived macrophages cocultured with MSCs in 2D on TCPS compared to 3D Carbylan-GSX. Data are shown as mean expression $\pm$ SD. Statistical significance of $p < 0.05$ is indicated by *.

There was higher CD206 expression among the 2D TCPS with BM- and AT-MSCs ($96.7\% \pm 1.2\%$, $96.85\% \pm 0.5\%$, $p > 0.1$) and Carbylan-GSX controls ($97.7\% \pm 1.5\%$). Additionally, we found lower CD206 expression in the TCPS alone and 3D MSC-hydrogel constructs ($81.6\% \pm 14.6\%$ TCPS, $81\% \pm 10.0\%$ hydrogel + BM-MSC, $87.5\% \pm 7.7\%$ hydrogel + AT-MSC, $89\% \pm 4.6\%$ hydrogel + VF-MSC, $p > 0.1$) though this was not statistically significant.

Expression of HLA-DR is lower on cells cocultured with MSCs regardless of culture material

The expression of HLA-DR was greatest in the macrophage population cultured on TCPS alone ($90.2\% \pm 5.9\%$), which was significantly higher than all of the other culture conditions. There was lower expression observed in macrophages cultured on the hydrogel alone compared to TCPS ($64.8\% \pm 9.1\%$ vs $90.2\% \pm 5.9\%$, $p < 0.05$). Furthermore, there was a significant decrease in HLA-DR expression in macrophages cultured in the presence of MSCs, regardless of culture material, with an average rate of about 25% ($28.5\% \pm 2.1\%$ 2D BM-MSCs, $20.4\% \pm 14.9\%$ 2D AT-MSCs, $24.4\% \pm 15.3\%$ hydrogel + BM-MSCs, $29.7\% \pm 16.3\%$ hydrogel + AT-MSCs, $23.6\% \pm 7.6\%$ hydrogel + VF MSCs, $p < 0.01$ compared to TCPS alone or hydrogel alone, $p > 0.1$ among the MSC groups).

Discussion

Inflammation is mediated by blood monocyte-derived or tissue-resident macrophages recruited to the site of tissue injury, be it from acute mechanical forces such as in trauma or surgery, infectious agents, or chronic underlying pathology. In later stages and after resolution of acute inflammation, stromal cells are being recruited, which may lead to fibrosis, scar formation, and organ dysfunction. Currently available therapeutic options to address soft tissue injury include watchful waiting, temporizing measures, such as topical antibiotics, growth factors, or anti-inflammatory applications, or surgical debridement. However, none of these methods are an ideal means to heal the injured tissue, and thus new strategies are being developed to not only replace wounded skin or connective tissues but also to regenerate

the native tissue lost.²⁷ One can anticipate that this task will require multi-modality therapy to mediate both the inflammatory resolution and tissue reparative phases of wound healing.

Bioengineered scaffolds could provide a microenvironment that allows for nutrient diffusion as well as biochemical, physical, and cellular stimuli that guides proliferation, differentiation, and migration of implanted or tissue resident cells. While many of these scaffolds are clinically used without cells for soft tissue augmentation or repair, such as collagen or HA, there is increasing interest in combining these ECM-based scaffold materials with cells for *in vivo* tissue engineering strategies. Biomaterials, such as MSC-hydrogel constructs, hold great promise in tissue engineering, but before the initiation of clinical trials, further studies are needed to investigate their potential interactions with human immune cells. This study demonstrates an *in vitro* model for investigating the immune response of human peripheral blood monocyte-derived macrophages when cocultured with human MSCs seeded in a hydrogel scaffold. Such studies are crucial in developing more effective biomaterial and cell-based therapies.

There are several factors associated with a material scaffold that can influence cell differentiation or function, including chemical composition (hydrophobicity, chemical stimuli, or added pendant chains), scaffold geometry (2D vs. 3D), and spatial relationship (cell concentration and porosity).^{28–30} In a similar manner, MSCs have been shown to modulate immune cell function through a variety of paracrine and juxtacrine effects, though these interactions are not completely understood. Over the past two decades there have been an increasing number of injectable fillers based on chemically modified biomaterials to use for soft tissue wound repair.¹ The next generation of sECM-based materials, such as the Carbylan-GSX investigated in this study, is of potential interest because it allows for an injectable cell-seeded construct that is capable of cross-linking *in situ* for local delivery in wound healing and other tissue engineering strategies.^{31–33}

In this study, we developed a novel coculture system to investigate the potential immunomodulatory and anti-inflammatory properties of such cell-hydrogel constructs. In particular, we determined the phenotype of macrophages cultured with BM- and AT-MSCs with and without an sECM scaffold. We found that the immunophenotypes of macrophages were different among the four different culture conditions, indicating an effect of both the sECM scaffold as well as MSC influence. CD14⁺/CD16⁺ monocytes are a subpopulation of circulating monocytes in peripheral blood in normal steady state; however, there is a drastic increase in CD14⁺/CD16⁺ cells in both acute and chronic inflammatory conditions.^{34,35} Such pro-inflammatory monocytes are responsible for the release of tumor necrosis factor- α , interleukin (IL)-1, and IL-12, among other cytokines involved in acute phase inflammation as well as frustrated phagocytosis and foreign body response.^{36,37} An additional marker of inflammation is HLA-DR expression on monocytes facilitating antigen presentation to T lymphocytes and a robust pro-inflammatory response.^{36,37} Monocyte expression of HLA-DR is used as an assessment of immune status and it is thought that a decrease in circulating HLA-DR⁺ monocytes is a gauge of a compensatory anti-inflammatory response

in systemic illness.³⁷ Alternatively, increased expression of the surface marker CD206 is an indicator of an anti-inflammatory phenotype.^{16,38,39}

Previous work with our novel HA-based sECM scaffold established *in vitro* and *in vivo* biocompatibility with MSCs.^{24,31} As CD14⁺ monocytes differentiated in the presence of our MSC-hydrogel constructs *in vitro*, there were no signs of toxicity to the monocytes in culture or induction of a pro-inflammatory phenotype. In fact, the immunophenotype of CD14⁺ macrophages at the end of the 7-day coculture could be characterized as an anti-inflammatory profile, based on low CD16, high CD206, and low HLA-DR expression. Macrophages cultured on Carbylan-GSX with MSCs expressed lower CD16 and HLA-DR with higher expression of CD206, indicating the least inflammatory profile overall. A unique aspect of this work is the allogeneic nature of the cells in that the macrophages were derived from different donors than the MSCs and that all six MSC lines used were isolated from different donors. Others have shown that while biologic scaffolds can induce an anti-inflammatory macrophage expression, adding a differentiated cellular component will induce a proinflammatory or M1 phenotype *in vivo*.^{40,41}

It is often difficult to predict how the *in vitro* outcomes will manifest into physiological responses, particularly in wound healing and biomaterial interactions. Translational, *in vitro* work such as this offers a controlled and manipulatable study environment to investigate the interactions between allogeneic cells, a biomaterial scaffold, and the specific cell type key in regulating these interactions. The statistically significant differences in macrophage surface marker expression among the treatment groups may contribute to understanding the undoubtedly complex mechanisms associated with MSCs in wound healing. Whether or not these differences will translate into physiological outcomes such as improved tissue regeneration or construct “take” can be hypothesized based on this study and warrants further investigation. This work communicates several interesting findings, including (1) the development of a coculture technique using an sECM scaffold to encapsulate MSCs in 3D during monocyte-derived macrophage development; (2) a direct comparison of BM and AT-derived MSCs as well as with a well-studied VF fibroblast cell line sharing similar functional characteristics to MSCs; (3) evidence that our sECM hydrogel scaffold induces an anti-inflammatory surface marker expression independent of 3D cell encapsulation; and (4) evidence supporting the benefit of an MSC-sECM construct for engineering strategies to address both tissue inflammation and repair. *In vitro* and *in vivo* work such as this is necessary in accelerating the translation of cell-based therapies from basic science to relevant, well-designed clinical trials.

Acknowledgments

This work was supported in part by NIH/NIDCD Grant R01 DC4336, R01 DC4336 S1 (S.L. Thibeault), NIH/NHLBI HL081076 K08, W81XWH-09-1-0532 (P. Hematti), NIH/NIDCD T32 Voice Science Training Grant DC009401 (S.N. King), NIH T32 Physician-Scientist Training Grant CA009614 (S.E. Hanson), and AAO-HNSF Resident Grant (S.E. Hanson). We thank Glen Levenson, Ph.D., Department of Surgery University of Wisconsin–Madison, for statistical assistance.

Sources of funding: NIDCD–NIH grants R01 DC4336, R01 DC4336S1, T32 DC009401; NHLBI–NIH grant K08 HL081076; DoD W81XWH-09-1-0532; and AAO-HNSF CORE grant.

Disclosure Statement

No competing financial disclosures exist. The authors have no conflicts of interest to report.

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Received: December 12, 2010

Accepted: May 09, 2011

Online Publication Date: June 17, 2011