Published in final edited form as:

Integr Biol (Camb). 2018 December 19; 10(12): 747–757. doi:10.1039/c8ib00132d.

An Adhesion Based Approach for the Detection of Esophageal Cancer

Mahboubah S. Noori^a, Evan S. Streator^a, Grady E. Carlson^a, David S. Drozek^b, Monica M. Burdick^{a,c,d}, and Douglas J. Goetz^{a,c,e}

^aDepartment of Chemical and Biomolecular Engineering, Ohio University, Athens, Ohio 45701

^bDepartment of Specialty Medicine, Ohio University, Athens, Ohio 45701

^cBiomedical Engineering Program, Ohio University, Athens, Ohio 45701

^dEdison Biotechnology Institute, Ohio University, Athens, Ohio 45701

Abstract

Esophageal cancer has a 5-year survival rate of ~20%. This dismal prognosis is due, in part, to the fact that esophageal cancer often presents at a late stage. Thus, there is a critical need for assays that enable the early detection of cancerous tissue within the esophagus. The luminal surface of the esophagus expresses signature molecule(s) at sites of transformation providing an avenue for the development of *in situ* assays that detect neoplastic growth within the esophagus. An attractive approach, receiving increased attention, is the endoscopic administration of particles conjugated with ligands to signature molecules present on transforming tissue. Detection of the particles within the esophagus, post-washing, would indicate the presence of the signature molecule and thus transforming tissue. In this work, we utilized cancerous and normal esophageal cells to provide *in vitro* proof of principle for this approach utilizing ligand-conjugated microspheres and demonstrate the need, and provide the framework for, engineering this technology. Specifically, the study (i) reveals selective increased expression of signature molecules on cancerous esophageal cells relative to normal cells; (ii) demonstrates selective binding of ligand-conjugated microspheres to cancerous esophageal cells relative to normal cells; (iii) demonstrates that the selective recognition of cancerous, relative to normal esophageal cells, is highly dependent on the biophysical design of the assay; and (iv) advocates utilizing the knowledge from the field of cell adhesion as a guide for the effective development of ligand-conjugated particle-based schemes that seek to detect esophageal oncogenesis *in situ*.

Graphical Abstract

^eAddress correspondence to: Douglas J. Goetz, Biomedical Engineering Program, Department of Chemical and Biomolecular Engineering, Ohio University, Athens, OH 45701 dougohio@gmail.com.

Conflicts of Interest

There are no conflicts to declare.

Selective recognition of esophageal cancer cells via ligand conjugated microspheres in an endoscopic adhesion assay

Introduction

The 5-year overall survival rate for esophageal cancer is at or below 20%¹. This bleak statistic is due to the aggressive nature of the cancer and the fact that esophageal cancer often presents at a late stage². In regard to the latter cause, it is likely that early detection would increase the 5-year survival rate, as it has for more common cancers such as colon and breast cancers. Thus, early detection of esophageal cancer is an important diagnostic goal [e.g.^{3–6}].

Changes in the biochemistry of esophageal transforming tissue, relative to normal tissue^{3–5,7–14}, provide an opportunity to develop assays that detect esophageal cancer at an early stage *in situ*. Indeed, several studies demonstrate that molecular constructs (i.e. ligands) cognate to biochemical targets selectively expressed on transforming esophageal tissue can be utilized to identify pathological tissue [e.g.^{4–6,15,16}]. Thus, a promising diagnostic approach is the utilization of ligand-target molecule recognition to selectively identify transforming tissue within the esophagus. In designing such schemes, arguably one of the most important aspects to consider are the factors that govern the selective recognition of pathological tissue. These factors can be divided into those that are fixed (e.g. the identity and surface density of the target signature molecules) and those that can be varied to optimize selective recognition. Among the controllable variables, perhaps the most important are those that define the design of the detection construct and the physical environment under which the detection construct and the target signature molecule-bearing tissue interact.

The detection construct could simply be the targeting ligand itself (e.g. a peptide^{5,16}) or it could be a particle conjugated with the ligand¹⁵. The ligand-conjugated particle could be a nanoparticle¹⁵ or could range from 10s to 100s of microns in diameter since the lumen of the esophagus is ~2 cm¹⁷. Utilization of particles, in particular particles in the micron range, has several salient features including the facts that such particles (i) can often be detected by bright field microscopy, (ii) allow a relatively large volume of imaging material (e.g. a fluorophore) to be carried per particle which can enhance detection [the volume scales with the particle radius cubed ($V \propto r^3$)], (iii) exhibit limited, if any, non-specific uptake by cells thus reducing non-specific signal and off target distribution, (iv) are relatively easy to manipulate experimentally, and (v) are easily washed out subsequent to detection. Thus,

other investigators have focused on the use of conjugated particles (nano and micron sized) for detection and characterizing of transforming tissue within the GI tract^{3,15,18,19}.

The physical environment under which the recognition takes place will be determined by the mode of delivery of the targeting construct which is intertwined with the detection scheme. Arguably, the easiest and perhaps cheapest way to introduce and detect ligand-conjugated particles is during an endoscopic procedure which is being performed during a clinical investigation. The above considerations combined, lead us to focus on advancing ligand-conjugated microparticle-based detection of transforming tissue in the esophagus using an endoscope for introduction and detection of the ligand-bearing particles. Specifically, the present work provides *in vitro* proof of principle for this approach and highlights the opportunity for, and need to, “engineer” such schemes to create a powerful *in situ* assay for the detection of transforming tissue within the esophagus.

Materials and Methods

Cell culture

OE19 human esophageal adenocarcinoma and OE21 human esophageal squamous cell carcinoma cells were obtained from Sigma-Aldrich (St. Louis, MO) and cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-Glutamine (Lonza; Basel, Switzerland), 10% non-heat inactivated FBS (VWR; Radnor, PA) and 1% penicillin/streptomycin (Lonza) at 37°C and 5.0% CO₂. Control cells were human esophageal epithelial cells (HEsEpiC) from ScienCell Research Laboratories (Carlsbad, CA) and cultured in Epithelial Cell Medium-2 (EpiCM-2, ScienCell) consisting of 500 ml basal medium, 5 ml epithelial cell growth supplement-2 (ScienCell), and 5 ml penicillin/streptomycin solution (ScienCell) at 37°C and 5.0% CO₂. HEsEpiC cells were cultured in poly-L-lysine-coated (ScienCell) tissue culture treated flasks (2 µg/cm²).

Flow cytometric analysis

Surface molecule expression on esophageal cancer cells and normal cells was evaluated using indirect single-color immunofluorescence and flow cytometry. In brief, cells were harvested with TrypLE Express (GIBCO; Gaithersburg, MD). Harvested cells were resuspended to 10⁷ cells/mL in Dulbecco's Phosphate Buffered Saline (DPBS) with Ca²⁺ or Mg²⁺ (Thermo Fisher Scientific; Waltham, MA) supplemented with 2% fetal bovine serum (FBS). Separate aliquots containing ~ 2 × 10⁵ cells were prepared, washed and treated with primary antibodies to various antigens (SLe^a, SLe^x, HECA-452 antigen, CD44, CD44v3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v7/8, CD44v10, VCAM-1, ICAM-1, and CD65s) or the appropriately matched isotype control and incubated on ice for 30 minutes. Mouse anti-SLe^a (KM231; IgG₁) monoclonal antibody (mAb) was obtained from EMD Millipore (Darmstadt, Germany). Mouse anti-human CD44 (515; IgG₁), rat anti-human cutaneous lymphocyte antigen (HECA-452; IgM), and mouse anti-human SLe^x (CSLEX-1; IgM) mAbs were purchased from BD Biosciences (San Jose, CA). Mouse anti-human mAbs against variant isoforms of CD44, [v3 (VFF-327; IgG₁), v4 (VFF-11; IgG₁), v5 (VFF-8; IgG₁), v6 (VFF-7; IgG₁), v7 (VFF-9; IgG₁), v7/8 (VFF-17; IgG_{2b}), and v10 (VFF-14; IgG₁)] were obtained from AbD Serotec (Raleigh, NC) Mouse anti-human ICAM-1 (IgG_{2b})

and anti-human VCAM-1 (1.G11B1; IgG₁) mAbs were purchased from EMD Millipore (Temecula, CA) and Ancell Corporation (Stillwater, MN), respectively. Mouse anti-human CD65s (VIM-2; IgM) mAb was obtained from An Der Grub Bio Research GmbH (Vienna, Austria). Matched isotype control for the mAbs to SLe^a, CD44, CD44v3, CD44v4, CD44v5, CD44v6, CD44v7, CD44v10, and VCAM-1 was purified mouse IgG₁ (mIgG). Purified mouse IgM was utilized as an isotype matched control for mouse anti-human SLe^x and CD65s mAbs while mouse IgG_{2b} was used for the mouse anti-human ICAM-1 and CD44v7/8 mAbs. The isotype control for rat HECA-452 mAb was purified rat IgM. All isotype controls were obtained from Southern Biotech (Birmingham, AL). After the incubation, the cells were washed and treated with the appropriate species matched FITC-conjugated secondary polyclonal antibody (pAb) (Southern Biotech) and incubated on ice for 30 minutes. Antibodies were diluted in, and washes were done with, 2% FBS/DPBS solution. The final wash was with DPBS and subsequently cells were resuspended in 100 μ L DPBS containing 1% paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA). Samples were immediately analyzed using a FACS Aria Special Order Research Product flow cytometer/sorter (BD Biosciences) and FlowJo software. Data are presented on a 5-decade log scale. For statistical analyses, the mean fluorescence intensity (MFI) of the population of cells treated with the mAb of interest and the isotype matched control were determined. The MFIs, from replicate experiments, were used in the statistical analyses.

Recombinant chimeric mouse and human E-selectin-human IgG₁ Fc constructs were obtained from R&D systems (Minneapolis, MN) and used for flow cytometric analysis to investigate the presence of E-selectin ligands on OE19 and HEsEpiC cells. To investigate the calcium dependence of E-selectin binding, recombinant E-selectin-IgG chimeras were separately diluted in two different buffers; chimera buffer [1% bovine serum albumin (BSA) in DPBS with Ca²⁺ or Mg²⁺] and negative control buffer containing EDTA [1% BSA and 20 mM EDTA in DPBS without Ca²⁺ or Mg²⁺]. Suspensions of OE19 and HEsEpiC cells were treated with recombinant murine E-selectin IgG₁ chimera (rmE-Sel), recombinant human E-selectin IgG₁ chimera (rhE-Sel) or human IgG₁ (hIgG) (Sigma-Aldrich), followed by a biotinylated rabbit anti-human IgG Fc polyclonal secondary antibody (Thermo Fisher Scientific, Waltham, MA) and finally phycoerythrin (PE) conjugated streptavidin (SA) (BD Biosciences). Each treatment was for 30 minutes on ice and followed by washes in chimera or negative control buffers. Subsequently, samples were analyzed using flow cytometry as described in the above paragraph.

Ligand-conjugated microsphere preparation

Ligand-conjugated microspheres were prepared in a manner similar to that previously described^{20,21}. 10 μ m protein-A conjugated and 106–125 μ m polystyrene microspheres were obtained from Bangs Laboratories (Fishers, IN) and Polysciences (Warrington, PA), respectively. First, both types of microspheres (5 $\times$ 10⁶ microspheres/mL) were washed twice with 1x tris buffered saline (TBS) (Thermo Fisher Scientific) pH 4 and then once in DPBS with Ca²⁺ and Mg²⁺. The microspheres were washed and incubated in TBS pH 8.2 containing either 50 μ g/mL of rmE-Sel, rhE-Sel chimera (R&D Systems), anti-CD44v5, mIgG, or hIgG at room temperature for 60 minutes with end over end agitation (note that the protein-A microspheres were blocked with 1% BSA in DPBS for 30 minutes prior to

incubation with the rmE-Sel, rhE-Sel chimera, or hIgG). Subsequently, the microspheres were washed and incubated with blocking buffer (DPBS containing 1% BSA) for 60 minutes at room temperature prior to use in the adhesion or flow cytometric assays. The resulting ligand-conjugated microspheres were analyzed by flow cytometry (for the 10 μ m diameter microspheres) or fluorescence microscopy (for the 106–125 μ m diameter microspheres) to verify the presence of ligand on the microspheres. To do so, separate aliquots of 10 or 106–125 μ m rmE-Sel, rhE-Sel, or hIgG microspheres, containing $\sim 2 \times 10^5$ of microspheres each, were treated (30 minutes on ice) with FITC-labeled F(ab')₂ goat anti-human IgG pAb (Jackson Immunoresearch), PE conjugated mouse anti-human CD62E mAb, or PE conjugated rat anti-mouse CD62E mAb (BD Bioscience) diluted in 1% BSA/DPBS. PE conjugated mouse IgG1 κ and rat IgG2 α k were used as isotype controls for the anti-human CD62E and anti-mouse CD62E mAbs, respectively. FITC-labeled goat anti-mouse IgG (H +L) pAb (Southern Biotech) was used to evaluate the presence of adsorbed anti-CD44v5 and mIgG on the 106–125 μ m anti-CD44v5 and mIgG treated microspheres. Subsequently, microspheres were washed twice with 1% BSA/DPBS, once with DPBS and resuspended in 100 μ L DPBS. 10 μ m microspheres were immediately analyzed using a FACSaria Special Order Research Product flow cytometer/sorter and FlowJo software. Data are presented on a 5-decade log scale. 106–125 μ m polystyrene microspheres were analyzed with a Nikon Microphot-SA fluorescent microscope.

Parallel plate laminar flow assay

The Glycotech (Gaithersburg, MD) parallel plate flow chamber was utilized to probe the selective adhesion of the microspheres to esophageal cancer cells relative to normal cells under fluid shear. Details of the operation have been given previously²². In brief, the parallel plate flow chamber was loaded with a slide supporting a cell monolayer. To prepare cell monolayers, 100 μ L of the harvested OE19 or HEsEpiC cells ($\sim 10^4$ cells) were placed into a 6 mm silicon ring (Unisyn Technologies; Hopkinton, MA) affixed to a 35-mm cell culture dish (Corning; Corning, NY). The dishes were incubated at 37°C and 5.0% CO₂ overnight prior to the adhesion assay. Once the flow chamber was loaded with a substrate, the assembly was placed on a Nikon Eclipse TE300 inverted microscope equipped with a Hitachi video camera to observe interaction of the microspheres with the cell monolayers throughout the entire assay period and for later off-line analysis. For the attachment assay, a suspension of rmE-Sel, rhE-Sel, or hIgG microspheres (3×10^5 microspheres/ml) in perfusion buffer (DPBS with Ca²⁺ or Mg²⁺ supplemented with 1% BSA) was introduced into the parallel plate flow chamber for 2 minutes at a shear stress of 0.5 dynes/cm². The number of microspheres that attached to the cell monolayers over the two minute time period was quantified from the recorded videos. For the detachment assay, the suspension of microspheres was introduced, and the flow was stopped after 2 minutes. The microspheres were allowed to settle and interact with the cell monolayers for a set amount of time (2 minutes). Subsequently, the flow was reinitiated at 0.5 dynes/cm² and the shear stress doubled every 30 seconds up to 32 dynes/cm². The number of bound particles was determined at the end of each shear stress interval.

Endoscopic adhesion assay

An endoscopic adhesion assay was employed to simulate the introduction of the ligand-conjugated microspheres during an endoscopic procedure. First, OE19, OE21, or HEsEpiC cells were plated on Lab-Tek II chamber slides (Thermo Fisher Scientific) and incubated at 37°C and 5.0% CO₂ overnight prior to the adhesion assay. Before starting the procedure, the cell monolayer was washed with perfusion buffer. Subsequently, a suspension of ligand-conjugated microspheres in perfusion buffer were perfused over the cell monolayer via the biopsy channel of an Olympus endoscope that has been threaded with Tygon tubing. The Tygon tubing was blocked with 1% BSA in DPBS for 30 minutes before starting the procedure. Once the microspheres had been delivered to the cell monolayer, the flow was stopped and the microspheres were allowed to incubate with the cells for 2 minutes. To remove unbound microspheres, the cell monolayer was washed via the auxiliary water channel of the endoscope. Subsequently, the number of bound microspheres was recorded. The camera at the distal tip of the scope projected the image/video to the computer throughout the entire assay period and recorded videos were used for later off-line analysis. Note that the pulsed delivery, followed by an incubation, and then a wash mimics how we envision this assay would work clinically.

Statistics

All results were evaluated using a one-way ANOVA coupled with post hoc Dunnett test. Results with p values less than 0.05 were considered significantly different. All error bars represent the standard error of the mean (SEM).

Results

Sialofucosylated glycans appear to have a higher expression on OE19 adenocarcinoma, relative to a normal, esophageal cell line

We first sought to identify signature surface molecules, i.e. surface molecules that are present or elevated on cancerous cells but not on normal cells, using the esophageal OE19 adenocarcinoma and HEsEpiC normal cell lines. Previous studies have suggested that the sialofucosylated glycans SLe^a and SLe^x are often upregulated on cancer cells/tissue including esophageal (e.g. ^{4,7,23–25}). Thus, we characterized the expression of SLe^a, SLe^x, and HECA-452 (a mAb to sialofucosylated glycans ²⁶) antigen on the cell lines using flow cytometric analysis. In addition, we assayed for the expression of CD65s (VIM-2) (a sialylated carbohydrate), VCAM-1 (CD106) ¹⁰, ICAM-1 (CD54) ¹³, CD44 ^{14,27}, and CD44 isoforms (e.g. ^{28,29}) since these have been reported to be altered on cancerous esophageal tissue.

As shown in Figure 1, HEsEpiC cells treated with anti-SLe^a (KM231), anti-SLe^x (CSLEX-1), HECA-452 and anti-CD44 (515) exhibited no shift, slight shift, moderate shift, and large shift in fluorescence intensity relative to cells treated with an isotype matched control. In contrast, OE19 cells treated with the same mAbs, exhibited dramatic shifts when treated with anti-SLe^a, anti-SLe^x, and HECA-452 but no shift when treated with anti-CD44. In addition to the above, we investigated the relative expression of VCAM-1, ICAM-1, CD65s and the CD44 variants; v3, v4, v5, v6, v7, v7/8, v10. As shown in Table 1, none of

these moieties appeared to be selectively expressed on the OE19 cell line. ICAM-1, CD65s and CD44 did appear to be expressed at a higher level on the HEsEpiC cell line compared to the OE19 cell line.

Flow cytometric analysis reveals that E-selectin chimeras exhibit preferential binding to OE19 cells relative to normal HEsEpiC esophageal cells

Based on the above results, sialofucosylated glycans appear to be the best target for selective identification of OE19 adenocarcinoma cells relative to normal cells. It is known from selectin biology that E-selectin is a ligand for SLe^a and SLe^x ²⁶. Additionally, HECA-452 recognizes a broad class of sialofucosylated glycans including SLe^x ²⁶ and, while not necessarily a function blocking mAb for SLe^x binding to E-selectin ³⁰, is a marker for the ability of a cell to be recognized by E-selectin ³¹. These observations led us to pursue E-selectin as a targeting ligand for discernment between OE19 adenocarcinoma and normal esophageal cell lines. We first sought to determine if E-selectin would exhibit preferred binding to the cancer cell line. For this, we used the human and murine extracellular E-selectin constructs fused to human IgG which we term rhE-Sel and rmE-Sel. We used both the human and murine versions of E-selectin since others have reported differences in the binding of these constructs to carbohydrate moieties ³². As shown in Figure 2A, OE19 cells treated with either the rmE-Sel or rhE-Sel exhibited a significant shift compared to the negative control OE19 cells treated with hIgG indicating that the E-selectin chimeras were bound to the OE19 cells. In contrast, HEsEpiC cells treated with either the rmE-Sel or rhE-Sel exhibited no shift compared to HEsEpiC cells treated with hIgG indicating that the E-selectin chimeras did not recognize the HEsEpiC cells (Figure 2B). The binding of the chimeras to the OE19 cells was not observed in the absence of Ca²⁺ and Mg²⁺ (Figure 2C), suggesting that the binding is specific since E-selectin binding is known to depend on Ca²⁺ and Mg²⁺ ³³. Note that the histograms for HEsEpiC were unchanged when Ca²⁺ and Mg²⁺ was removed (Figure 2D compared to Figure 2B) again suggesting that the E-selectin chimeras did not recognize the HEsEpiC cells.

Coupling a human and murine E-selectin-IgG chimera to polystyrene microspheres

Based on the above results, we next sought to generate E-selectin conjugated microspheres as potential detection constructs. The E-selectin chimeras were non-covalently coupled to separate sets of 10 µm diameter protein-A microspheres via the protein-A on the microspheres and the human Fc domain of the E-selectin chimeras. Control microspheres were generated by coupling human IgG to a separate set of protein-A microspheres. The resulting rmE-Sel, rhE-Sel, and hIgG microspheres were analyzed by flow cytometry to gain insight into the success of the coupling. As shown in Figure 3A, rmE-Sel, rhE-Sel, and hIgG microspheres treated with FITC-labeled anti-human IgG antibody exhibited a pronounced increase in fluorescence relative to unconjugated microspheres treated with the same FITC-labeled antibody and rmE-Sel, rhE-Sel or hIgG microspheres not treated with the FITC-labeled antibody. These observations indicate the rmE-Sel, rhE-Sel, and hIgG constructs were successfully coupled to the microspheres. rmE-sel and rhE-sel were recognized by a mAb to E-selectin (Figure 3B) demonstrating that the E-selectin portion of the chimera was accessible. The E-selectin mAbs did not recognize hIgG microspheres (Figure 3B) demonstrating that the recognition of the E-selectin microspheres by the mAbs was specific.

E-selectin microspheres can discriminate between OE19 adenocarcinoma and normal esophageal cells under flow conditions and the selectivity of the adhesion depends on the biophysics of the assay

We next investigated whether the E-selectin microspheres could discriminate between the OE19 adenocarcinoma and normal cell lines in an adhesion assay that has a fluid dynamic component. For this we utilized the parallel plate flow chamber that is routinely used in cell adhesion assays. We first measured the rate of attachment of the microspheres to the two cell monolayers. As shown in Figure 4, the level of attachment of the rhE-sel and rmE-sel microspheres to the OE19 cells was significantly greater than the level of attachment to the HEsEpiC cells. Additionally, the level of attachment of the rhE-sel and rmE-sel microspheres to the OE19 cells was significantly greater than the level of attachment of the IgG microspheres to the OE19 cells. Clearly, the E-selectin microspheres can discern OE19 adenocarcinoma from normal esophageal cells. The selectivity (ratio of E-selectin microspheres on OE19 cells to E-selectin microspheres on HEsEpiC cells) was ~88 for rmE-Sel and ~56 for rhE-Sel. Additionally, we observed that rmE-Sel microspheres were more firmly bound to the OE19 cells relative to the rhE-Sel microspheres with the rhE-Sel microspheres exhibiting a rolling adhesion as opposed to a firm adhesion that was observed for the rmE-Sel microspheres. These latter observations suggest that changes in the biochemistry of the targeting scheme, e.g. changing the ligand targeting chemistry from rhE-Sel to rmE-Sel which have quantitative sequence similarity, can have a significant effect on the results of the selectivity assay.

We next asked whether changing the delivery mode of the assay would yield different results. In particular, a clinical assay utilizing an endoscope to deliver and detect the microspheres would likely be run in “detachment mode” wherein the microspheres are delivered to the tissue in question, allowed to incubate with the tissue and then the tissue washed to remove unbound microspheres. Thus, we next ran the adhesion assay in detachment mode. For this we introduced a suspension of microspheres, discontinued the flow for 2 minutes, restarted the flow at 0.5 dynes/cm² and increased the shear stress by 0.5 dynes/cm² every 30 seconds up to 32 dynes/cm². At each shear stress, the number of microspheres adherent to the cell monolayers was determined. The results of this analysis are presented in Figure 5.

As revealed in Figure 5, the rmE-Sel (Figure 5A) and rhE-Sel microspheres (Figure 5B) were again able to discriminate between OE19 and HEsEpiC cells. This is seen by comparing the level of adhesion observed on OE19 cells (black circles in each panel) to that observed on the HEsEpiC cells (grey squares in each panel). The selectivity was a function of fluid shear and ranged, for the rmE-Sel microspheres, from ~1.1 at 0.5 dynes/cm² to ~6.8 at 32 dynes/cm² and, for the rhE-Sel microspheres, from ~3.6 at 0.5 dynes/cm² to infinity at shear stress 8 dynes/cm². Note that for the rhE-Sel microspheres, the selectivity approaches infinity at shear stress 8 dynes/cm² due to the number of adherent microspheres on the HEsEpiC cells going to zero.

Combined, these results demonstrate that the ligand-conjugated microsphere approach has significant promise for yielding a novel assay for the detection of cancerous esophageal tissue *in situ* and that the power of this approach is dependent on the biochemical and

biophysical design of the assay. In the next section we describe a technique that can be used in the rational development of this technology.

Endoscopic adhesion assay – a tool to develop an *in situ* diagnostic for transforming tissue within the esophagus

The data presented in the above sections clearly demonstrates that microspheres conjugated with E-selectin exhibit selective recognition of OE19 adenocarcinoma, relative to normal, esophageal cells. This result suggests that particles conjugated with E-selectin might be a starting point for a probe which could be used to enhance *in situ* detection of adenocarcinoma during an endoscopic procedure. To provide further proof of principle for this approach, we utilized an endoscopic adhesion assay. Specifically, we used an endoscope to expose OE19 and HEsEpiC cell lines to rhE-Sel, rmE-Sel, or hIgG (negative control) microspheres. In this case we used 106–125 µm diameter microspheres to allow facile detection of the microspheres via the endoscope camera. In preliminary studies, immunofluorescence using anti-E-selectin and anti-hIgG antibodies revealed that the E-selectin chimeric constructs and human IgG were coupled to the microspheres (data not shown). Lab-Tek II chamber slides were prepared with OE19 or HEsEpiC monolayers. The microspheres were delivered to the cell monolayers via the biopsy channel of the endoscope that had been threaded with Tygon tubing. Once introduced, via pressure exerted by a syringe, the pressure was equilibrated and the microspheres allowed to interact with the cellular substrates for 2 minutes. Subsequently, the monolayers were washed via the axillary water channel of the endoscope. The endoscope camera was utilized to observe and record the monolayer throughout this process. Note that the pulsed delivery, followed by an incubation, and then a wash mimics how we envision this assay would work clinically.

As shown in Figure 6, significantly more E-selectin microspheres adhered to the OE19 cells relative to the HEsEpiC cells. The selectivity was infinite for rmE-Sel (due to no microspheres being present on the HEsEpiC monolayers) and ~440 for rhE-sel. The adhesion appeared to be specific as few, if any, hIgG microspheres bound to either cell lines. Combined, this data provides proof of concept for a ligand-conjugated microparticle-based detection scheme that could be developed into a method for *in situ* detection of transforming tissue within the esophagus and provides an important tool that can be used to develop this technology.

An endoscopic adhesion assay that targets CD44v5 discriminates between OE21 squamous cell carcinoma and normal esophageal cell lines

To provide further proof of concept, we sought to discriminate between OE21 squamous cell carcinoma and normal esophageal cells in the endoscopic adhesion assay. We first characterized the surface chemistry of OE21 cells using flow cytometric analysis. This analysis revealed that OE21 cells do not appear to express SLe^a or the HECA-452 antigen and express only a modest level of SLe^x (data not shown). The OE21 cells do express a significant level of CD44v5 while HEsEpiC do not (Figure 7). Therefore, we chose to discriminate between OE21 and HEsEpiC esophageal cells via anti-CD44v5 microspheres in the endoscopic adhesion assay. As shown in Figure 8, a significantly greater level of adhesion occurred between anti-CD44v5 microspheres and the OE21 cells compared to the

level of adhesion that occurred between anti-CD44v5 microspheres and HEsEpiC cells. The selectivity of anti-CD44v5 microspheres for OE21 cells was ~ 500. The adhesion appeared to be specific as few, if any, mIgG microspheres bound to either of the cell lines. Thus, the endoscopic adhesion assay can discriminate between normal esophageal cells and two types of esophageal cancer cells (OE19 adenocarcinoma and OE21 squamous cell carcinoma).

Discussion

There has been an increasing effort to exploit neoplastic changes in the biochemistry of the apical surface of the esophagus as a means to detect and characterize precancerous or cancerous tissue [e.g. ^{4-6,15,16}]. For example, Bird-Lieberman et al.⁴ demonstrated the feasibility of using lectins to discern dysplasia in Barrett's esophagus. Li et al.⁵ identified a peptide (SNFYMPL) that exhibits a high level of binding to dysplastic tissue and this same group reported on another peptide, ASYNYDA, that exhibited augmented binding to esophageal neoplasia⁶. Similarly, Zhou et al.¹⁶ identified a peptide (SRRPASFRtare) that ligates the extracellular domain of FGFR2 and exhibits elevated binding to high grade dysplasia and esophageal squamous cell carcinoma specimens. Work has also been done with ligand-conjugated nanoparticles. For example, a sophisticated study demonstrated endoscopic identification and characterization, utilizing multiple surface-enhanced Raman scattering nanoparticles, of xenograft cancerous material implanted within the rat esophagus¹⁵. Regardless of the details of the detection scheme, these approaches, as well as the use of micron-sized particles as outlined in the present work, are unified in that the success of the assays will be determined by the ability of the detection construct(s) to discern between healthy and transforming tissue. This success is ultimately dictated by the biophysics governing the adhesion/binding of the constructs with the tissue.

Thus, in this study, we initially utilized OE19 adenocarcinoma cells and the normal esophageal cell line HEsEpiC to: (i) provide proof of principle for such an assay that utilizes ligand-conjugated microspheres, (ii) highlight the importance of the biophysics underlying these assays and (iii) engender an engineering approach in the development of these assays. We first screened the OE19 and normal cell lines, via flow cytometric analysis, for surface markers that would allow discrimination between the cancerous and normal cells. We found that CD44 was present on the normal cells but not the OE19 cells (Figure 1). This result is in general agreement with Darlavoix et al.'s work ¹⁴ that found reduction in CD44 expression with progression in esophageal cancer. Specifically, they observed higher expression of CD44 in low, relative to high, grade dysplasia in specimens of a patient with esophageal adenocarcinoma ¹⁴. Second, in this study we found that sialofucosylated epitopes (as identified by HECA-452 and mAbs to SLe^a and SLe^x) were present at a higher level of OE19 cells compared to the normal cell line (Figure 1). This result is in line with Bird-Lieberman et al. ³⁴ which identified SLe^a as a marker associated with the progression of Barrett's esophagus to high grade dysplasia or esophageal adenocarcinoma.

The above findings motivated the use of E-selectin as a ligand to discriminate between OE19 adenocarcinoma and normal esophageal cells since E-selectin recognizes SLe^x, SLe^a ²⁶ and HECA-452 reactive cells ³¹. To our knowledge, there have been few, if any, studies probing the binding of E-selectin constructs to esophageal cells. We found that microspheres

conjugated with E-selectin can discriminate between cancerous adenocarcinoma (OE19) and normal esophageal cells (HEsEpiC) under flow conditions including conditions that mimic the clinical setting (Figures 4, 5, 6). To provide further proof of principle and to illustrate the use of another signature molecule and ligand, we utilized the OE21 squamous cell carcinoma cell line and CD44v5 as the target (Figure 7) in the endoscopic adhesion assay (Figure 8). The assay did indeed discriminate between OE21 and normal esophageal cells (Figure 8) with a very high level of selectivity (~500). Taken together, the results strongly suggest that a ligand-conjugated microsphere approach has significant promise for yielding a novel assay for the detection of transforming esophageal tissue *in situ*. It is insightful to note that the targeting chemistry utilized for OE19 (sialofucosylated glycans and E-selectin) and OE21 (CD44v5 and anti-CD44v5) were distinct. Additionally, in preliminary studies we did try to detect OE21 with E-selectin chimeras but were unable to distinguish between OE21 and normal esophageal cells using this ligand. Further, the OE19 cells lacked detectable cell surface expression of CD44v5 (Table 1). These observations highlight the need for a multi-target approach to develop a “robust” detection scheme as advocated by Wang et al.¹⁵ with nanoparticles. The differential expression of targets in heterogeneous tumors and thus the need to consider multi-ligand targeting further motivates an engineering approach in the development of these detection schemes.

While the above results provide proof of principle, it is important to recognize that the most effective assays will be ones that can distinguish early transforming tissue (as opposed to overt carcinomas) from normal tissue. We are currently exploring early transforming cells and normal cells in this assay. We anticipate that the differences between transforming and normal cells will be subtler than between overt carcinoma (e.g. OE19 and OE21) and normal cells. In this regard, our observations regarding the biophysics of the assay become important. Specifically, we observed that, (i) the physical environment under which the ligand-conjugated microspheres interact with the cells significantly influences the selectivity of the assay (e.g. the selectivity of the detachment assay was a function of fluid shear in Figure 5), and (ii) the chemistry used as the targeting ligand, even when the chemistry is quite similar (rmE-sel vs rhE-sel), significantly influences the results (e.g. compare the adhesion of rhE-sel versus rmE-sel microspheres to OE19 cells in Figure 6C). These observations reveal that the operative biophysics dictates the performance of the assay and, most importantly, demonstrate the opportunity to tune the biophysics to achieve high selectivity.

Fortunately, there is ample related work that can guide the tuning of the biophysics to reveal subtle differences in tissue biochemistry. Specifically, the particle-based *in situ* assays explored here are analogous to the well-studied phenomena of cellular adhesion to vascular endothelium under blood flow conditions. A plethora of experimental and theoretical studies have elucidated the biophysical underpinnings of cell adhesion and a host of tools have been developed to study cell adhesion (e.g.^{22,35–39}). These efforts have demonstrated that adhesion is an intricate balance between an adhesive force that arises between the adhering cell and the endothelium and a disruptive force exerted on the adhering cell by the flow of the blood. Whether adhesion occurs, and the nature of the adhesion, depends on this overall force balance which is dictated by many factors including the size of the cell, the surface density of the cognate adhesion molecules, the operative fluid dynamics (e.g. level of fluid

shear), and the kinetic and tensile properties of the bonds mediating the adhesion³⁷. Similarly, the selective adhesion of the ligand-conjugated particles to transformed, relative to healthy tissue, in esophageal oncogenesis detection schemes will be dictated by a rather complex force balance between the adhesive force that arises between the particles and the tissue and the disruptive force exerted by the flow of fluid surrounding the particles. The adhesive force will consist of non-specific forces between the particles and the tissue³⁵, which may be similar for transformed and normal tissue, and specific forces mediated by the ligand – target molecule adhesive bonds³⁵ that are ideally minimal on healthy tissue and maximal on transformed tissue. The disruptive fluid force is due to the fluid stream introduced through the endoscope or the fluid passing through the esophagus during swallowing (see next paragraph). Note that the disruptive force is an important driver of the differentiation between adhesive and non-adhesive microparticles. Clearly, the knowledge, tools and analyses of cell adhesion can be utilized to efficiently “engineer” selective recognition of transforming tissue and achieve a highly effective ligand-conjugated particle-based assay for the *in situ* detection of oncogenesis within the esophagus.

Finally, we note that in this work we focused on the detection of the particles via an endoscope. Other detection modalities, including those that use an external device to detect the particles, could ultimately be used (e.g. γ -radiation, MRI, ultrasound). In these scenarios, the particles may be introduced via deglutition, i.e. by having the patient swallow a suspension of ligand-conjugated particles containing a moiety that could be detected externally. In this case, a force balance would again be germane to the assay with the disruptive force arising due to fluid transport through the esophagus as opposed to the fluid force imparted by the endoscope. Previous work, which provides an understanding of fluid flow during deglutition (e.g.⁴⁰), could be combined with models of cell adhesion (e.g.³⁷) and work focused on oral administration of pharmaceutical agents⁴¹, to provide a foundation to rationally develop an *in situ* detection assay where the ligand-conjugated particles are introduced via deglutition.

Acknowledgments

This work was supported by The National Institutes of Health grant: R15 CA161830-01 (MMB/DJG)

References

1. Browse the SEER Cancer Statistics Review 1975–2014, https://seer.cancer.gov/csr/1975_2014/browse_csr.php?sectionSEL=8&pageSEL=sect_08_table.22.html, (accessed December 24, 2017).
2. Nair KS, Naidoo R and Chetty R, J. Clin. Pathol, 2005, 58, 343–351. [PubMed: 15790695]
3. Chan LW, Wang YN, Lin LY, Upton MP, Hwang JH and Pun SH, Bioconjug. Chem, 2013, 24, 167–175. [PubMed: 23273065]
4. Bird-Lieberman EL, Neves AA, Lao-Sirieix P, O'Donovan M, Novelli M, Lovat LB, Eng WS, Mahal LK, Brindle KM and Fitzgerald RC, Nat. Med, 2012, 18, 315–321. [PubMed: 22245781]
5. Li M, Anastassiades CP, Joshi B, Komarck CM, Piraka C, Elmunzer BJ, Turgeon DK, Johnson TD, Appelman H, Beer DG and Wang TD, Gastroenterology, 2010, 139, 1472–1480. [PubMed: 20637198]
6. Sturm MB, Joshi BP, Lu S, Piraka C, Khondee S, Elmunzer BJ, Kwon RS, Beer DG, Appelman HD, Turgeon DK and Wang TD, Sci. Transl. Med, 2013, 5, 184RA61.

7. Oshiba G, Kijima H, Tanaka H, Kenmochi T, Himeno S, Kise Y, Nishi T, Chino O, Shimada H, Abe Y, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M and Makuuchi H, *Int. J. Oncol*, 2000, 17, 701–705. [PubMed: 10995880]
8. Aarsen LAKV, Leone DR, Ho S, Dolinski BM, McCoon PE, LePage DJ, Kelly R, Heaney G, Rayhorn P, Reid C, Simon KJ, Horan GS, Tao N, Gardner HA, Skelly MM, Gown AM, Thomas GJ, Weinreb PH, Fawell SE and Violette SM, *Cancer Res.*, 2008, 68, 561–570. [PubMed: 18199553]
9. Kwon J, Lee T-S, Lee HW, Kang MC, Yoon H-J, Kim J-H and Park JH, *Int. J. Oncol*, 2013, 43, 1523–1530. [PubMed: 24042193]
10. Heidemann J, Maaser C, Lügering A, Spahn TW, Zimmer KP, Herbst H, Rafiee P, Domschke W, Krieglstein CF, Binion DG and Kucharzik TF, *Int. J. Oncol*, 2006, 28, 77–85. [PubMed: 16327982]
11. Hong L, Han Y and Brain L, *Expert Opin. Ther. Targets*, 2013, 17, 1179–1185. [PubMed: 23855932]
12. Izmirli M, Bayram I, Senol S and Ilhan M, *Int. J. Hematol. Oncol. UHOD Uluslararası Hematol. Onkol. Derg.*, 2009, 19, 28–35.
13. Alasehirli B, Oguz E, Oksuzler E, Koruk I, Oztuzcu S, Ozkara E, Karakok M, Erbagci AB and Demiryurek AT, *Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med*, 2014, 35, 4907–4912.
14. Darlavoix T, Seelentag W, Yan P, Bachmann A and Bosman FT, *Virchows Arch.*, 2009, 454, 629–637. [PubMed: 19396460]
15. Wang YW, Kang S, Khan A, Bao PQ and Liu JTC, *Biomed. Opt. Express*, 2015, 6, 3714–3723. [PubMed: 26504623]
16. Zhou J, He L, Pang Z, Appelman HD, Kuick R, Beer DG, Li M and Wang TD, *Oncotarget*, 2017, 8, 87095–87106. [PubMed: 29152066]
17. Kuo B and Urma D, *GI Motil. Online*, 2006, DOI:10.1038/gimo6.
18. Kirui DK, Rey DA and Batt CA, *Nanotechnology*, 2010, 21, Art. No.: 105105.
19. Iftimia N, Iyer AK, Hammer DX, Lue N, Mujat M, Pitman M, Ferguson RD and Amiji M, *Biomed. Opt. Express*, 2012, 3, 178–191. [PubMed: 22254178]
20. Goetz DJ, Greif DM, Ding H, Camphausen RT, Howes S, Comess KM, Snapp KR, Kansas GS and Luscinskas FW, *J. Cell Biol*, 1997, 137, 509–519. [PubMed: 9128259]
21. Carlson GE, Martin EW, Shirure VS, Malgor R, Resto VA, Goetz DJ and Burdick MM, *PLOS ONE*, 2017, 12, e0173747. [PubMed: 28282455]
22. Gopalan PK, Jones DA, McIntire LV and Smith CW, eds. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM and Strober W, *Current Protocols in Immunology J. Wiley*, New York, 1996, pp. 7.29.1–7.29.23.
23. Goetz DJ, Brandley BK and Hammer DA, *Ann. Biomed. Eng.*, 1996, 24, 87–98. [PubMed: 8669721]
24. Goetz DJ, Ding H, Atkinson WJ, Vachino G, Camphausen RT, Cumming DA and Luscinskas FW, *Am. J. Pathol*, 1996, 149, 1661–1673. [PubMed: 8909255]
25. Makino T, Shimada Y, Maeda M, Komoto I and Imamura M, *Oncol. Rep*, 2001, 8, 981–985. [PubMed: 11496302]
26. Berg EL, Robinson MK, Mansson O, Butcher EC and Magnani JL, *J Biol Chem*, 1991, 266, 14869–14872. [PubMed: 1714447]
27. Zhao J-S, Li W-J, Ge D, Zhang P-J, Li J-J, Lu C-L, Ji X-D, Guan D-X, Gao H, Xu L-Y, Li E-M, Soukiasian H, Koeffler HP, Wang X-F and Xie D, *PloS One*, 2011, 6, e21419. [PubMed: 21731740]
28. Gotoda T, Matsumura Y, Kondo H, Ono H, Kanamoto A, Kato H, Watanabe H, Tachimori Y, Nakanishi Y and Kakizoe T, *Gut*, 2000, 46, 14–19. [PubMed: 10601048]
29. Nozoe T, Kohnoe S, Ezaki T, Kabashima A and Maehara Y, *J. Cancer Res. Clin. Oncol*, 2004, 130, 334–338. [PubMed: 14872339]
30. Kummitha CM, Shirure VS, Delgadillo LF, Deosarkar SP, Tees DF, Burdick MM and Goetz DJ, *J. Immunol. Methods*, 2012, 384, 43–50. [PubMed: 22820001]
31. Wagers AJ, Stoolman LM, Craig R, Knibbs RN and Kansas GS, *J Immunol*, 1998, 160, 5122–9. [PubMed: 9590264]

32. Ni Z and Walcheck B, *Immunol. Lett*, 2007, 108, 179–182. [PubMed: 17222460]
33. Bevilacqua MP, *Annu Rev Immunol*, 1993, 11, 767–804. [PubMed: 8476577]
34. Bird-Lieberman EL, Dunn JM, Coleman HG, Lao-Sirieix P, Oukrif D, Moore CE, Varghese S, Johnston BT, Arthur K, McManus DT, Novelli MR, O'Donovan M, Cardwell CR, Lovat LB, Murray LJ and Fitzgerald RC, *Gastroenterology*, 2012, 143, 927–935.e3. [PubMed: 22771507]
35. Cozens-Roberts C, Quinn JA and Lauffenburger DA, *Biophys J*, 1990, 58, 107–125. [PubMed: 2166596]
36. Lawrence MB and Springer TA, *Cell*, 1991, 65, 859–873. [PubMed: 1710173]
37. Hammer DA and Apte SM, *Biophys J*, 1992, 62, 35–57.
38. Alon R, Hammer DA and Springer TA, *Nature*, 1995, 374, 539–542. [PubMed: 7535385]
39. Shinde Patil VRS, Campbell CJ, Yun YH, Slack SM and Goetz DJ, *Biophys J*, 2001, 80, 1733–1743. [PubMed: 11259287]
40. Li M, Brasseur JG and Dodds WJ, *Am. J. Physiol*, 1994, 266, G525–543. [PubMed: 8178991]
41. Drumond N and Stegemann S, *Colloids Surf. B Biointerfaces*, 2018, 165, 303–314. [PubMed: 29524806]

Insight box

Esophageal cancer often presents at a late stage and has a dismal prognosis. Early detection of this disease will significantly increase the chances of long-term survival. During the development of esophageal cancer, the mosaic of molecules present on the lining of the esophagus changes. There is an increasing focus on exploiting these changes for the detection of esophageal cancer. By utilizing methodology from the field of cell adhesion, we provide a proof of principle for a ligand-conjugated microparticle-based *in situ* diagnostic, highlight the importance of the underlying biophysics in dictating the success of this diagnostic approach, and provide an avenue to engineer an assay that could ultimately provide for *in situ* detection of oncogenesis within the esophagus.

Figure 1. Characterization of adhesion molecule expression on esophageal adenocarcinoma OE19 cells and normal HEsEpiC cells.

OE19 (top panels) and HEsEpiC (bottom panels) cells were labeled with anti-SLe^a (KM231; first column) mAb, anti-SLe^x (CSLEX-1; second column) mAb, HECA-452 (third column), anti-CD44 (515; fourth column) mAb (solid gray lines), or isotype control (dashed black lines) and analyzed by flow cytometric analysis. OE19 cells treated with mAbs to SLe^a and SLe^x, and HECA-452 showed a significant shift in mean fluorescence intensity (MFI) compared to cells treated with an isotype control ($n = 3$; $p < 0.05$). In contrast, OE19 cells treated with CD44 exhibited no statistically significant shift in fluorescence compared to cells treated with the isotype control. HEsEpiC did not show a significant shift in MFI when treated with a mAb to SLe^a compared to the isotype control but did exhibit a significant shift when treated with a mAb to CD44 compared to the isotype control ($n = 3$; $p < 0.05$). HEsEpiC appeared to exhibit a slight shift in MFI when treated with a mAb to SLe^x and a moderate shift when treated with HECA-452 compared to the isotype controls. However, each of these latter two observations (SLe^x and HECA-452) did not reach statistical significance when replicate experiments were averaged and analyzed ($n=3$; $p=0.1$ for SLe^x and $p=0.08$ for HECA-452). Presented histograms are representative of three separate experiments.

Figure 2. Fluid phase E-selectin chimeras bind to esophageal adenocarcinoma OE19 cells but not normal HEsEpiC cells.

Separate aliquots of OE19 cells (A and C) and HEsEpiC cells (B and D) were treated with recombinant murine or human E-selectin – Fc chimeras (rmE-Sel or rhE-Sel) or human IgG (hIgG) and analyzed via flow cytometric analysis in the presence (A and B) or absence (C and D) of divalent cations. In the presence of divalent cations, OE19 cells (A), but not HEsEpiC cells (B), treated with either rmE-Sel (solid grey line) or rhE-Sel (solid black line) exhibited a significant increase in fluorescence relative to cells treated with human IgG (dashed line) ($n=3$; $p<0.05$). The shift in fluorescence observed for the OE19 cells was markedly attenuated in the absence of divalent cations (C). The fluorescence profile for HEsEpiC cells was nearly identical with or without divalent cations (B vs D).

Figure 3. Flow cytometric analyses of 10 µm diameter microspheres conjugated with recombinant human or murine E-selectin chimeras or human IgG.

10 µm diameter protein-A microspheres were coupled with rmE-Sel, rhE-Sel, or hIgG and analyzed via flow cytometric analysis. (A) rmE-Sel, rhE-Sel and hIgG microspheres treated with the FITC-labeled antibody to human IgG exhibited a significant increase in fluorescence (black line) relative to unconjugated microspheres treated with the same antibody (dashed gray line) and ligand-conjugated microspheres not treated with the antibody to IgG (dashed black line) demonstrating that the ligands were successfully coupled to the microspheres. (B) The rmE-Sel, rhE-Sel and hIgG microspheres were treated with a PE labelled antibody to murine E-selectin, human E-selectin or both antibodies, respectively and analyzed by flow cytometry. The antibodies to E-selectin recognized rmE-Sel (solid light gray line) and rhE-Sel (solid dark gray line) on rmE-Sel and rhE-Sel microspheres, respectively, but did not recognize the hIgG microspheres. The shift in the fluorescence of the rmE-sel and rhE-sel microspheres treated with PE labelled antibodies to E-selectin was substantial relative to the rmE-sel and rhE-sel microspheres treated with PE-labelled isotype control (dashed gray line) and the rmE-sel and rhE-sel microspheres not treated with a PE antibody or isotype control (dashed black line). Results are representative of three separate experiments.

Figure 4. An attachment adhesion assay utilizing E-selectin microspheres discriminates between esophageal adenocarcinoma OE19 cells and normal HEsEpiC cells.

(A) 10 µm diameter protein-A microspheres conjugated with rmE-Sel, rhE-Sel, or hIgG (negative control) were perfused over separate monolayers of OE19 or HEsEpiC cells in a parallel plate flow chamber. The number of attached ligand-conjugated microspheres was determined and divided by the area of the field of view under observation. The attachment of the E-selectin microspheres to OE19 cells was significantly higher than the attachment of E-selectin microspheres to the normal HEsEpiC cells and the attachment of hIgG microspheres to OE19 cells. Results are the average of three separate experiments. # $p < 0.05$ attachment of rmE-Sel or rhE-Sel microspheres to OE19 cells relative to HEsEpiC cells. * $p < 0.05$ attachment of rmE-Sel or rhE-Sel microspheres to OE19 cells relative to attachment of hIgG microspheres to OE19 cells. (B) Representative images of rmE-Sel microspheres adherent to OE19 (top image) or HEsEpiC (bottom image) cells. Red arrows highlight the 10µm rmE-Sel microspheres.

Figure 5. A detachment adhesion assay utilizing E-selectin microspheres discriminates between esophageal adenocarcinoma OE19 cells and normal HEsEpiC cells.

10 μ m diameter protein-A microspheres conjugated with (A) rmE-Sel or (B) rhE-Sel were perfused over separate monolayers of OE19 (black line with circles) or HEsEpiC (gray line with squares) cells in a parallel plate flow chamber. Once introduced into the flow chamber, the flow was stopped, and the microspheres allowed to interact with the cell monolayers for 2 minutes. Subsequently, the flow was reinitiated in step-wise increments of shear and the number of adherent microspheres per unit area at the end of the increment determined. In both cases, the level of adhesion observed on the OE19 cells is markedly greater than that observed on the HEsEpiC cells.

Figure 6. E-selectin microspheres discriminate between OE19 esophageal adenocarcinoma cells and normal cells in an endoscopic adhesion assay.

106–125 μ m polystyrene microspheres passively conjugated with rmE-Sel, rhE-Sel, or hIgG were perfused over monolayers of OE19 or HEsEpiC cells grown on Lab-Tek II chamber slides. Microspheres were delivered to the cellular substrates through the biopsy channel of an endoscope that had been threaded with Tygon tubing. Once introduced, the microspheres were allowed to interact with the cell monolayers for 2 minutes. Subsequently, the monolayers were washed via the axillary water channel of the endoscope. The endoscope camera was used to observe and record the interaction of the microspheres with the monolayers. (A) Images generated using the endoscope camera and (B) images captured through the microscope eyepiece revealed that a significantly greater level of adhesion occurred between E-selectin conjugated microspheres and the OE19 cells compared to the level of adhesion that occurred between E-selectin microspheres and HEsEpiC cells. Additionally, the level of adhesion between E-selectin microspheres and the OE19 cells was significantly greater than that between the hIgG microspheres and OE19 cells. (C) The number of adherent ligand-conjugated microspheres was determined and divided by the area of the field of view under observation. Results shown are the average of three separate experiments. # $p < 0.05$ rmE-Sel or rhE-Sel microspheres on OE19 versus HEsEpiC cells. \$ $p < 0.05$ rmE-Sel or rhE-Sel microspheres on OE19 cells versus hIgG microspheres on OE19 cells. * $p < 0.05$ rmE-Sel microspheres on OE19 cells versus rhE-Sel microspheres on OE19 cells.

Figure 7. The expression of CD44v5 is elevated on OE21 squamous cell carcinoma cells relative to normal esophageal cells.

OE21 (left panel) and HEsEpiC (right panel) cells were labeled with anti-CD44v5 mAb (solid gray lines) or isotype control (dashed black lines) and analyzed by flow cytometry. OE21 cells treated with anti-CD44v5 mAb showed a significant shift in mean fluorescence intensity (MFI) compared to cells treated with the isotype control ($n=3$; $p<0.05$). In contrast, HEsEpiC cells treated with anti-CD44v5 mAb exhibited no significant shift in fluorescence compared to cells treated with an isotype control. Histograms are representative of three separate experiments.

Figure 8. Anti-CD44v5 microspheres discriminate OE21 squamous cell carcinoma cells from normal esophageal cells in an endoscopic adhesion assay.

106–125 μm polystyrene microspheres passively adsorbed with anti-CD44v5 or mIgG were perfused over a monolayer of OE21 or HEsEpiC cells in an endoscopic adhesion assay. (A) Images generated using the endoscope camera and (B) images captured through the microscope eyepiece revealed that a significantly greater level of adhesion occurred between anti-CD44v5 microspheres and the OE21 cells compared to the level of adhesion that occurred between anti-CD44v5 microspheres and HEsEpiC cells. Additionally, the level of adhesion between anti-CD44v5 microspheres and the OE21 cells was significantly greater than that between the mIgG microspheres and OE21 cells. (C) The number of adherent microspheres was determined and divided by the area of the field of view under observation. Results shown are the average of three separate experiments. # $p < 0.05$ anti-CD44v5 microspheres on OE21 versus HEsEpiC cells. \$ $p < 0.05$ anti-CD44v5 microspheres on OE21 cells versus mIgG microspheres on OE21 cells.

Table 1.
Characterization of surface moieties present on esophageal adenocarcinoma OE19 and normal esophageal HEsEpiC cell lines using flow cytometric analysis.

Each cell line was treated with a mAb to the antigen listed on the left and analyzed by flow cytometric analysis. None of these moieties appeared to be selectively expressed on the OE19 cell line. ICAM-1, CD65s and CD44 appeared to be expressed to a higher level on the HEsEpiC cell line compared to the OE19 cell line (+ Significant shift in mean fluorescence intensity (MFI), +/- Slight shift in MFI, and – No shift in MFI compared to the isotype control).

	OE19	HEsEpiC
VCAM-1	–	–
ICAM-1	–	+
CD65s (VIM-2)	–	+/-
CD44 (515)	–	+
CD44v3	–	–
CD44v4	–	–
CD44v5	–	–
CD44v6	–	–
CD44v7	–	–
CD44v7/8	–	–
CD44v10	–	–