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Effect of avidin-like proteins and biotin modification on mesenchymal stem cell adhesion

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Abstract

The avidin-biotin system is a highly specific reaction that has been used in a wide range of biomedical applications, including surface modification and cell patterning. We systematically examined a number of avidin derivatives as the basis for a simple and cost effective tissue culture polystyrene substrate surface modification for human stem cell culture. Non-specific adhesion between human mesenchymal stem cells and various avidin derivatives, media conditions, and subsequent biotinylation reactions was quantified. We observed significant non-specific cell adhesion to avidin and streptavidin, indicating that previous observations using this system may be artifactual. Seeding of cells in serum free media, blocking with bovine-serum albumin, and the use of the avidin derivative Neutravidin were all necessary for elimination of background adhesion. Neutravidin conjugated with biotinylated bsp-RGD(15) peptide provided the most robust cell adhesion, as well as the greatest increase in cell adhesion over background levels.

Keywords

avidin; streptavidin; neutravidin; biotin; stem cells; mesenchymal stem cells; non-specific adhesion

Introduction

The interaction between avidin and biotin is highly specific, and has one of the highest known non-covalent bond strengths, with a dissociation constant of 5×10^{-14} M.[1] Due to the strength and specificity of this reaction, the avidin-biotin conjugation system has been used in the biological, biointerface, and sensor communities for a wide range of applications, including: western blots and ELISA assays;[2-5] protein and cell purification from solid substrates;[6] and, affinity chromatography.[7] The avidin/biotin scheme has also been used in a wide range of cell patterning studies, as the ability to create multiple structured layers is advantageous in these schemes. For example, patterns of biotin have been created using photolithographic processes with a novel resist, [8] patterns of streptavidin have been created by “scraping” off a protein resistant poly(ethylene glycol)

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(pEG) layer with a scanning probe,[9] and patterns of avidin or streptavidin have been microcontact printed onto polymeric substrates.[10, 11]

Avidin itself, a protein isolated from avian egg whites, is a tetrameric glycoprotein that can bind up to four biotin molecules, with each subunit binding individually to one biotin. Although avidin has been used in the aforementioned applications, the protein has been implicated in non-specific adsorption to substrates, binding to other biomolecules in bioassays, and adhesion to cell membranes due to avidin's glycosylation and net positive charge.[12] Therefore, a number of derivatives have been developed, including both streptavidin and neutravidin that eliminate the carbohydrate groups from the protein.[13] However, RYG amino acid sequences present on surface of the streptavidin molecule are thought to structurally mimic the ubiquitous cell adhesive RGD sequence found in many cell adhesion proteins, resulting in mammalian cell adhesion to the protein.[14] Neutravidin was designed to have a near neutral isoelectric point, further minimizing non-specific and electrostatic interactions.[15]

Despite the widespread use of avidin, and its derivatives, in the biointerface and biomaterials communities, to date no study has systematically examined the potential non-specific interactions between mammalian cells and various avidin derivatives, and subsequent biotinylation reactions. In this work, we examined peptide-modified biotin as a mimetic for the extracellular matrix proteins that stem cells exploit for adhesion.[16] Two deposition schemes were explored for conjugating peptides to the surface (Figure 1). In Scheme 1, the avidin (or analogue) was simply physisorbed onto a tissue culture treated polystyrene substrate (TCPS), followed by conjugation with a biotin-grafted molecule of interest. In Scheme 2, the substrate was first blocked with a biotinylated bovine serum albumin (BSA) to separate the avidin molecule from the surface and prevent potential denaturation caused by adsorption. Human mesenchymal stem cell adhesion to standard tissue culture polystyrene surfaces modified using both these schemes, presenting a number of avidin derivatives and biotinylated molecules of interest, was quantified.

Methods

Surface Preparation

A number of combinations of albumin blocking, media conditions, and avidin derivatives were deposited onto polystyrene surface in an attempt to minimize non-specific cell adhesion, and are listed in Table 1. Biotinylated BSA and avidin derived proteins were adsorbed from PBS (pH 7.2) at 0.1 mg/mL for 1hr followed by three rinses with PBS. Biotin-containing molecules were conjugated to the avidin derivatives for 30 mins at 0.1 mg/mL in PBS, and rinsed three times. For blocking experiments, the modified surfaces were exposed to a 1% solution of bovine serum albumin (BSA, Sigma) or BSA that had been inactivated for 45 minutes in at a 60C water bath (HIBSA) for 60 min. Surfaces were kept bathed in PBS until cell seeding. Avidin, streptavidin, and neutravidin were all purchased from Invitrogen (Carlsbad, CA). All peptides were purchased from American Peptides (Sunnyvale, CA). Biotin-pEG was purchased from Nanocs (New York, NY). Streptavidin-RGD was graciously donated by Patrick Stayton's lab at the University of Washington.[17]

A number of biotin peptides were studied on the surfaces prepared in this paper, including a 15 amino acid peptide containing the sequence RGD that had been derived from bone sialoprotein (biotin-CGGNGEPRGDTYRAY-NH₂, termed **biotin-bsp-RGD(15)**), and interacts with the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.[18-20] A peptide sequence termed AG-73 was also tested, consisting of the amino acid sequence biotin-RKRLQVQLSIRT, termed **biotin-AG-73** that is believed to interact with the heparin sulfate side chains of the Syndecan-I

transmembrane protein, and thought to provide a separate cell adhesive pathway to integrin binding.[18, 19, 21, 22]

Cell Culture and quantification

Human mesenchymal stem cells (Lonza, Walkersville, MD) were cultured in MSC growth medium (Lonza) containing 10% serum and 1% gentamicin. For adhesion experiments, cells were allowed to adhere for 4 hrs onto the surfaces in a 96 well plate at $\sim 10^4$ cells/well (100 μ L/well) in hMSC growth media containing serum (Lonza) or serum free DMEM (Gibco/Invitrogen, Carlsbad, CA) depending on experimental conditions. After 4 hrs, the cells were rinsed once with PBS and frozen for >24 hrs at -80°C , followed by quantification of the number of adhered cells using the CyQuant assay (Invitrogen, Carlsbad, CA) using a Gemini Spectramax fluorimeter (Molecular Devices, Menlo Park, CA). Standard curves to calculate cell density based on relative fluorescent units (RFUs) were generated by creating a stock solution of known cell density (counted on a hemocytometer), and performing serial dilutions before applying the same CyQuant assay.

Cells were examined on peptide-modified surfaces to assess morphology using a Nikon T300 in phase contrast mode. For this, cells were plated at 10^4 cells/cm² in either a 24 or 48 well plate on the desired surface in serum free media. Cells were allowed to attach for 1 hr before being rinsed with fresh media and imaged. The remaining cells were imaged again after 1d to visualize morphology of spread and proliferating cells.

Statistics

Each experiment was run in triplicate and analysis of variance (ANOVA) was conducted, and a value of $p < 0.05$ was used to determine significance. Differences among surfaces and media conditions were assessed using Tukey's honestly significant difference (Tukey HSD) post hoc pairwise comparison.

Results & Discussion

We initially examined the effect of both serum and heat inactivation of bovine serum albumin (HI-BSA) in a passivating blocking step on hMSC attachment to avidin and avidin/biotin-bsp-RGD(15) surfaces. Samples were constructed as shown in Scheme 1 of Figure 1: adsorption of avidin, followed by biotin-bsp-RGD(15), followed by normal BSA or HI-BSA. There were statistically significant differences between cells seeded in the presence of serum versus cells seeded without serum (Fig. 2). hMSC attachment to all the surfaces was greater in the presence of serum. For surfaces without serum and no avidin modification, blocking was sufficient to eliminate non-specific hMSC attachment. Heat inactivation of BSA had no influence non-specific cell attachment from serum free media. However, it was noted that avidin with and without biotin-bsp-RGD(15) resulted in the same degree of cell adhesion, suggesting non-specific attachment of the hMSCs to the avidin molecule was significant.

Due to the non-specific adhesion of hMSCs to avidin, a number of other avidin derivatives were examined to minimize non-specific interactions with hMSCs. Proteins were adsorbed to TCPS in the following order: the avidin or derivative (i.e., avidin, streptavidin, neutravidin, or streptavidin-RGD) followed by biotinylation (i.e., biotin-PEG or biotin-bsp-RGD(15)), and then blocking with standard BSA. Experiments were run in serum free media, defining 2000 cells/well as a median degree of cell adhesion. All surfaces without BSA blocking were at or above this level (Fig. 3). Blocking with BSA effectively reduced non-specific hMSC adhesion for certain negative control samples, notably background adhesion to neutravidin or neutravidin/biotin-pEG (Fig. 3). Addition of the biotin-bsp-

RGD(15) molecule significantly improved cell adhesion to neutravidin modified surfaces, while cell adhesion to streptavidin/biotin-bsp-RGD(15) surfaces did not show a significant difference from streptavidin only surfaces (Fig. 3). For a particular avidin derivative, the greatest difference in hMSC adhesion occurred between neutravidin/biotin-pEG and neutravidin/biotin-bsp-RGD(15).

To determine if adsorption to TCPS affected the function of streptavidin or neutravidin, we preadsorbed polystyrene surfaces with biotin-modified BSA prior to avidin derivative modification, as shown in Scheme 2 of Figure 1. Proteins were adsorbed in the following order: biotin modified BSA (biotin-BSA), followed by either streptavidin or neutravidin, followed by a biotin-bsp-RGD(15) or biotin-pEG, followed by blocking with BSA. Experiments were run in serum free media. Preincubation with biotin-BSA improved overall cell adhesion for streptavidin/biotin-bsp-RGD(15) modified surfaces, suggesting that physical adsorption of streptavidin to polystyrene has a deleterious effect the protein's ability to bind biotin (Fig. 4). For neutravidin, preincubation with biotin-BSA did not affect cell adhesion on RGD modified surfaces.

To demonstrate the use of the BSA blocked neutravidin system for rapidly studying mixed peptide interfaces, biotin-bsp-RGD(15) was compared to both a negative control peptide RGE and to biotin-AG-73, a peptide that engages with the heparin sulfate side groups of Syndecan-1 and has been implicated in human embryonic stem cell adhesion and proliferation.[16, 21] For this experiment, hMSCs were plated into 24 well culture plates and imaged at one hour and one day to examine both density and cell morphology, and the resulting images are shown in Figure 5. Cells attached and spread down to a 50-50 mixture of RGD and RGE, while at 40% RGD and below cell attachment was sporadic and the cells remained rounded on the surface (Figure 5d). In biotin-bsp-RGD(15) and biotin-AG-73 peptide mixtures, hMSCs were attached for all combinations of peptide, from 100% RGD to 100% AG-73. However, at 40% RGD and below, cells did not spread and form morphologies that were normally observed for hMSCs on surfaces with higher bsp-RGD(15) content. It was also observed that the cells formed colony-like morphology on the surface on substrates presenting AG-73 peptides (Fig. 5 h-m).

Conclusions

In this paper, the influence on cell adhesion for a variety of avidin derivatives and biotinylated peptides was examined. A range of avidin derivatives were adsorbed to tissue culture polystyrene, including avidin, neutravidin, streptavidin, and a streptavidin that had been modified to include cell adhesive RGD motif. Based on these data, the simplest avidin-based system for presenting cell binding peptides was determined to be a biotin-peptide conjugated to physisorbed neutravidin, followed by blocking with BSA. This peptide presenting system provided a high level of cell attachment when conjugated with biotin-bsp-RGD(15), while minimizing non-specific cell adhesion. This system also allowed for a variety of biotinylated molecules to be examined, including biotin-bsp-RGD(15) and biotin-AG-73 as cell adhesive ligands, and biotin-pEG and biotin-RGE as a blocking agent or negative control, respectively. Both blocking with BSA and removal of serum from the media were necessary to prevent background adhesion to the tissue culture polystyrene, regardless of the avidin/biotin scheme used, due to potential displacement of the avidin proteins from the surface by the Vroman effect or interactions between cell adhesive proteins in serum and avidin/streptavidin. The results presented are of particular importance to the wide range of studies that use the avidin/biotin system for generating micro/nanopatterns on cell culture substrates. The observed non-specific adhesion of human MSCs to both avidin and streptavidin, and the lack streptavidin activity when functionalization with a biotin-RGD, would preclude use of these systems for studies involving hMSCs and

possibly other stem cells. The use of neutravidin and proper blocking with pEG or BSA will be critical to future use of avidin/biotin in designing cell/materials interfaces, particularly ones designed to interact with human mesenchymal stem cells.

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Figure 1.

Avidin/biotin deposition schemes. In Scheme 1, the avidin protein (or derivative) was simply physisorbed to a polystyrene surface before conjugating with a biotin molecule of choice. In Scheme 2, a biotinylated-BSA molecule was first deposited, followed by avidin and a second biotin-linked molecule.

Figure 2.

Effect of heat inactivated BSA (HI-BSA) and serum on human MSC adhesion to various avidin derivatives adsorbed to TCPS. Samples were prepared as shown in Scheme 1 of Figure 1. The effect of BSA blocking was most pronounced on neutravidin surfaces. For a particular avidin derivative, the greatest difference in hMSC adhesion occurred between neutravidin/biotin-pEG and neutravidin/ biotin-bsp-RGD(15). Groups marked with different letters (A-E) are statistically significant ($p < 0.05$); ANOVA with TukeyHSD post-hoc test.

Figure 3.

The effect of BSA blocking on human MSC adhesion to various avidin derivatives in serum free media. Samples were prepared as shown in Scheme 1 of Figure 1. Groups marked with different letters (A-E) are statistically significant ($p < 0.05$); ANOVA with Tukey HSD post-hoc test.

Figure 4.

The effect of biotin-BSA on human MSC adhesion in serum free media. Proteins were adsorbed to TCPS as shown in Scheme 2 of Figure 1 ((+) **BSA-biotin**): biotin modified BSA (biotin-BSA), followed by either streptavidin or neutravidin, followed by a biotin-bsp-RGD(15) or biotinpEG, followed by blocking with BSA. Samples were prepared as shown in Scheme 1 of Figure 1 ((-) **BSA-biotin**). Groups marked with different letters (A-E) are statistically significant ($p < 0.05$); ANOVA with Tukey HSD post-hoc test.

Figure 5. Human MSCs attachment to different peptide-modified TCPS surfaces. Cells were attached on various mixtures of biotin-bsp-RGD(15) and RGE (a-d) or mixtures of biotin-bsp-RGD(15) and AG-73 (g-l) in serum free media. Controls of neutravidin or BSA blocked surfaces are shown in (e-f). (sb = 200 microns)

Table 1

Avidin and biotin derivatives tested.

Avidin Derivatives	Biotinylated molecules
Avidin	biotin-RGD
Streptavidin	biotin-RGE
Neutravidin	biotin-AG73
Streptavidin-RGD	biotin-pEG