Cell Adhesion to Unnatural Ligands Mediated by a Bifunctional Protein

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Abstract: This paper describes a molecular strategy to restore adhesion of cells to surfaces that otherwise do not present ligands that can mediate adhesion. The approach is based on a carbonic anhydrase fusion protein that binds benzenesulfonamides and that also includes the RGD peptide motif that can bind to cell-surface integrin adhesion receptors. In this way, the fusion protein can bind to a monolayer that presents the benzenesulfonamide ligand, thereby positioning the RGD peptide at the surface, where it can mediate the adhesion and spreading of cells. This strategy may provide a general method for promoting the adhesion of cells to non-natural surfaces or to defective biological matrices.

Introduction

Most cells are adherent and must attach and spread on a protein matrix in order to survive and carry out their functions. Most commonly, the integrin family of receptors mediates adhesion by binding to peptides within the matrix. In this way, the adhesion of cells to a given substrate can be promoted by either modifying the latter with ligands for an endogenous receptor or engineering the cell to introduce receptors that recognize an epitope on the surface.[1−4] Both routes have been explored for promoting tissue integration with medical devices and for treating the blistering diseases caused by laminin and collagen mutations that disrupt normal adhesion of the epidermis and dermis.[5] An unexplored route to promote cell adhesion when complementary receptors and ligands are not present would use an adaptor protein that can simultaneously bind to the cell-surface receptor and an epitope present on the substrate. In this paper we demonstrate that this strategy can restore cell adhesion to substrates that do not present ligands for the endogenous receptors. Like adhesion to a normal matrix, the adaptor protein-mediated adhesion is specific, and the polyvalent engagement of receptors is able to sustain the mechanical forces applied by the cell and support the assembly of a contractile cytoskeleton.

We use self-assembled monolayers (SAMs) that present ligands against a background of tri(ethylene glycol) groups as model substrates for cell adhesion. Previous work has shown that monolayers that present the RGD peptide ligand support the integrin-mediated adhesion and spreading of cells.[6] The glycol groups are an important component of the monolayers, as they prevent the nonspecific attachment of cells and therefore ensure that adhesion is mediated only by the immobilized
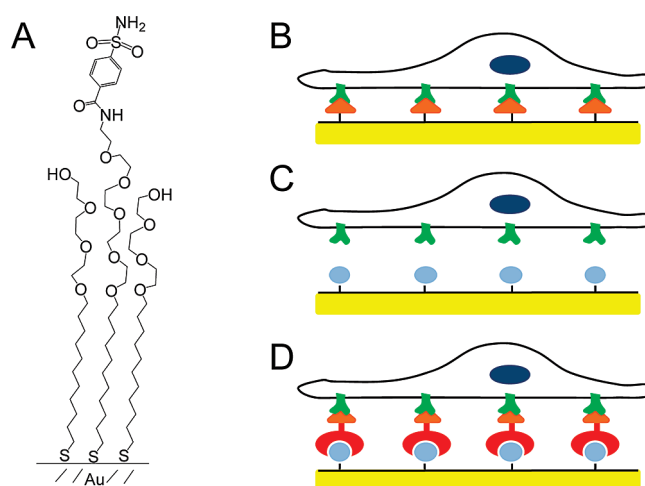

Figure 1. CHO cell adhesion to model substrates. (A) Schematic of benzenesulfonamide-presenting monolayer. (B) CHO cells adhere to RGD-presenting monolayers, but (C) fail to adhere to benzenesulfonamide surfaces. (D) CHO cells adhere to benzenesulfonamide surfaces in the presence of CA-RGD.

ligand.[2] In the present work, we characterized the adhesion of Chinese hamster ovary (CHO) cells on a monolayer presenting a benzenesulfonamide ligand (Figure 1A). The CHO cells display α5 and αv integrins on their surfaces, which interact with the canonical RGD tripeptide motif present in the matrix protein fibronectin (Figure 1B).[7] These cells are unable to attach to the benzenesulfonamide-terminated monolayer (Figure 1C). To restore adhesion to this monolayer, we designed and expressed a human carbonic anhydrase IV (hCAIV) protein that included at its carboxy-terminus a linker and the RGD motif (CA-RGD) (Figure 1D). Because carbonic anhydrase binds to benzenesulfonamide ligand with micromolar affinity,[8] we

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reasoned that the inclusion of this protein in the cell culture medium would result in its binding to the monolayer and therefore recruitment of the RGD motif to the surface, where it could mediate adhesion of the CHO cells. Ternary complex formation has been shown to be an efficient method of targeting antibodies to virus-infected cells.[9]

Our results demonstrate that cell adhesion to unnatural ligands can be induced in the presence of the adaptor protein. The observed cell adhesion phenotype was similar to the one observed on covalently modified RGD surfaces, suggesting that cells can exert the same contractile forces on both substrates. This work is significant as it adds a new method to engineer cell adhesion. This strategy is widely applicable and of special interest when surface modification or receptor introduction into a cell is impractical or impossible.

Materials and Methods

CA-RGD Construction and Expression. CA-RGD was amplified from a previous hCAIV gene with adaptor primers including *Nhe*I and *Bam*HI recognition sequences. The sequence of the forward primer, including an *Nhe*I recognition site followed by the first six codons of hCAIV, was 5′-ATATGCTAGCGCCGGTCTA-GAGTCACAC-3′. The reverse complementary sequence of the reverse primer, including a *Bam*HI recognition site followed by the last five codons of hCAIV, followed by the codons of the GGSGGGGSGGRGDS sequence and a stop codon, was 5′-A T T A G G A T C C T T A G C T G T C A C C G C G A C - C A C C G C T A C C A C C A C C A C C G C T A C C A C C G - GACTTTATCACCGT-3′. The polymerase chain reaction (PCR) consisted of an initial 1 min denaturation at 95 °C, followed by 30 cycles of 1 min 95 °C denaturation, 1 min 55 °C annealing, and 2.5 min of 73 °C extension. After 30 cycles, a final extension at 73 °C for 5 min followed by cooling at 4 °C was performed. The PCR products were cleaved with *Nhe*I and *Bam*HI, purified, and cloned in frame into the pET-11a vector (Novagen). Insertion of the hCAIV gene with the C-terminal linker and RGDS sequence was confirmed by DNA sequencing. For ease of purification, a six His tag was introduced in the N-terminus using a QuikChange site-directed mutagenesis kit (Stratagene). The forward primer had the sequence 5′-GATATACATATGGCTAGCCACCAC-CACCACCACCACGCCGGTCTAGACTCAC-3′. The reverse primer had the sequence 5′-GTGACTCTAGACCG-G C G T C G T C G T C G T C G T C G T G G T G G T G - GTGGGGTGGCTAGCCATATGTATATC-3′. The construct was amplified with the cycling parameters suggested in the product, using 18 cycles. Insertion of the His tag was verified with DNA sequencing. The construct was then transformed into *Escherichia coli* (strain Origami B DE3) cells for production of CA-RGD following standard procedures. For protein expression, single ampicillin-resistant colonies were grown in Luria−Bertani medium supplemented with 100 mg/mL ampicillin at 37 °C and 190 rpm overnight. A 1 mL aliquot of this culture was used to inoculate 1 L of Luria−Bertani medium supplemented with 100 mg/mL ampicillin and grown to an OD_{600} of 0.6. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the cells grew for 4 h at 37 °C and 190 rpm. Cultures were then centrifuged and pellets resuspended in 20 mL of phosphate-buffered saline (PBS) (10 mM phosphate, 150 mM NaCl, pH 7.0). Cells were sonicated for 10 min, and the suspension was centrifuged to remove cellular debris. The supernatant was applied to a TALON single step (Clontech) and incubated in ice for 30 min. After initial elution, the column was incubated with 10 mL of buffer A (25 mM TRIS, 1.5 mM $MgCl_2$, 5 mM NaCl, 1.25% Triton X)

on ice for 30 min. This step was performed twice. After elution, the column was incubated with 5 mL of buffer B (25 mM TRIS, 1.5 mM $MgCl_2$, 5 mM NaCl, 150 mM imidazole) for 10 min on ice and eluted. This fraction was concentrated using 10 kDa Amicon filtration units (Millipore) and extensively dialyzed against PBS (10 mM phosphate, 150 mM NaCl, pH 7.0) in 10 kDa dialysis cassettes. The purity of the protein was assessed with sodium dodecyl sulfide−polyacrylamide gel electrophoresis; protein concentration was measured using NanoDrop.

Peptide Synthesis. Ac-GRGDSC, Ac-GGRDGSC, Ac-GRGDS, and Ac-GGRDGS were synthesized manually following standard Fmoc peptide synthesis protocols using Fmoc-Rink amide MBHA resin. All peptides were purified by reverse-phase high-performance liquid chromatography using a C18 column (Waters) and characterized with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Preparation of Monolayers. Glass coverslips were sonicated for 30 min in deionized ultrafiltered (DIUF) water and 30 min in ethanol and then dried under a stream of nitrogen. Titanium (4 nm) and then gold (29 nm) were evaporated onto the coverslips using an electron beam evaporator (Boc Edwards) at a rate of 0.05−0.10 nm/s and at a pressure of 1 μTorr. The gold-coated coverslips were immersed in an ethanolic solution of either maleimide-terminated disulfide (2%) and tri(ethylene glycol)-terminated disulfide (98%) or amine-terminated disulfide (1%) and tri(ethylene glycol)-terminated disulfide (99%) and incubated overnight at room temperature. The total disulfide concentration was 1 mM. The substrates were washed with DIUF water and ethanol and then dried under a stream of nitrogen.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) measurements were performed using 1% benzenesulfonamide SAMs and CA-RGD following the methods described by Mrksich et al.[8]

Cell Adhesion Assay. Cysteine-terminated peptides were immobilized onto 1% maleimide-presenting SAMs by immersing the biochip in 1 mM peptide solution (TRIS buffer, pH 7.5) and incubating at 37 °C for 1 h. Benzenesulfonamide was immobilized by immersing a 1% amine-terminated SAM in a 1 mM benzenesulfonamide−NHS solution (1:1 DMSO:100 mM triethanol amine, pH 7) and incubating at room temperature for 30 min. The reaction was repeated three times to ensure completion. Substrates were rinsed with DIUF water and ethanol and dried under a stream of nitrogen. CHO K1 cells were detached from culture plates with 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)/2 mM ethylenediaminetetraacetic acid (EDTA) in PBS, rinsed with F-12K medium (10% fetal bovine serum (FBS), 1X penicillin/streptomycin), centrifuged, and resuspended in the same medium at a density of 60 000/mL. Cells were added to substrates in 24-well culture plates. For 1% benzenesulfonamide substrates, the cell suspension was supplemented with 10 μM CA-RGD. Cells were incubated at 37 °C and 5% CO_2 for 4 h. Upon completion of the incubation period, substrates were gently washed with warm PBS and imaged with a 20× objective of a Zeiss Axiovert 200 inverted microscope.

Immunostaining. CHO K1 cells were allowed to adhere and spread on substrates as described above. Substrates were washed gently with PBS, and permeabilized with 4% formaldehyde and 0.5% Triton-X for 30 min. Fixed and permeabilized cells were blocked in the presence of blocking buffer (1% bovine serum albumin, 5% goat serum, and 0.1% Triton-X) for 30 min. Substrates were then incubated with 1:1000 dilution of monoclonal antivinculin immunoglobin (IgG) (Sigma) for 30 min, followed by incubation with 1:400 dilution of phalloidin-AF488 and TR goat antimouse IgG and 1:5000 4′,6-diamidino-2-phenylindole (DAPI) for 30 min. After washing extensively with blocking buffer, the substrates were mounted with Aqua Poly/Mount (PolySciences Inc.). Fluorescent images of cells on substrates were acquired with a 100× objective of a Zeiss Axiovert 200 inverted microscope.

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Cell Adhesion Assay at Different Concentrations of CA-RGD. Benzenesulfonamide was immobilized by immersing a 1% amine-terminated SAM in a 1 mM benzenesulfonamide−NHS solution (1:1 DMSO:100 mM triethanol amine, pH 7) and incubating at room temperature for 30 min. The reaction was repeated three times to ensure completion. Substrates were rinsed with DIUF water and ethanol and dried under a stream of nitrogen. CHO K1 cells were detached from culture plates with 1 mM EGTA/2 mM EDTA in PBS, rinsed with F-12K medium (10% FBS, 1X penicillin/streptomycin), centrifuged, and resuspended in the same medium at a density of 60 000/mL. Cells were added to substrates in 24-well culture plates with different concentrations of CA-RGD. Cells were incubated with at 37 °C and 5% CO_2 for 4 h, followed by a gentle PBS wash. Adherent cells were immediately fixed and permeabilized with 4% formaldehyde and 0.5% Triton-X for 30 min. Fixed and permeabilized cells were stained with DAPI (1:5000) for 30 min and mounted with Aqua Poly/Mount (Poly-Sciences Inc.). Fluorescent images of cells' nuclei on substrates were acquired with a 20× objective of a Zeiss Axiovert 200 inverted microscope. The number of adherent cells per field of view was counted using Image J. Cells were counted in at least five fields for each substrate, and each experiment was repeated three times. Adhesion is reported as the average count of nuclei per field of view, using the standard error for error bars.

Cell Adhesion Inhibition Assay. Suspensions of CHO K1 cells (60 000/mL) were incubated with soluble inhibitors (Ac-GRGDS, Ac-GGRDGS, and benzenesulfonamide) for 10 min prior to addition to benzenesulfonamide substrates (in the presence of CA-RGD). Cells were incubated as described in the cell adhesion assay. After incubation, the substrates were washed gently with warm PBS, and adherent cells were immediately fixed and permeabilized with 4% formaldehyde and 0.5% Triton-X for 30 min. Fixed and permeabilized cells were stained with DAPI (1:5000) for 30 min and mounted with Aqua Poly/Mount (PolySciences Inc.). Fluorescent images of cells' nuclei on substrates were acquired with a 20× objective of a Zeiss Axiovert 200 inverted microscope. The number of adherent cells per field of view was counted using Image J. Cells were counted in at least five fields for each substrate, and each experiment was repeated three times. The degree of inhibition is reported as a percentage of cells that adhere relative to control experiments in the absence of soluble inhibitor. Standard error was used for error bars.

Results

CA-RGD Expression and Binding. The adaptor protein CA-RGD is composed of carbonic anhydrase IV (CAIV) followed by a linker, GGSGGGGSGG. The C terminal of the linker is terminated with an RGDS peptide that promotes cell adhesion. A His tag was introduced in the N terminal of the CAIV segment for ease of purification (Figure 2A). The binding of CA-RGD was characterized using SPR. SPR experiments demonstrated that CA-RGD binds to 1% benzenesulfonamide SAMs and fails to bind to 100% ethylene glycol SAMs (Figure 2B). These experiments showed that the expressed protein is active and that it binds selectively to benzenesulfonamide substrates.

CA-RGD Mediates Chinese Hamster Ovary Cell Adhesion and Cytoskeletal Structure Comparable to Ac-GRGDSC. We next examined if CA-RGD was capable of mediating CHO cell adhesion to benzenesulfonamide SAMs. As a control, we also seeded CHO cells on 1% Ac-GRGDSC substrates, onto which the RGD peptide had been covalently immobilized. CHO cells failed to adhere to 1% benzenesulfonamide substrates (Figure 3B). When the experiment was repeated using medium that was supplemented with CA-RGD (10 μM), the cells attached to the monolayer and assumed a well-spread morphology (Figure 3C). The cells were indistinguishable from those that attached to a control monolayer that directly presented the RGD peptide
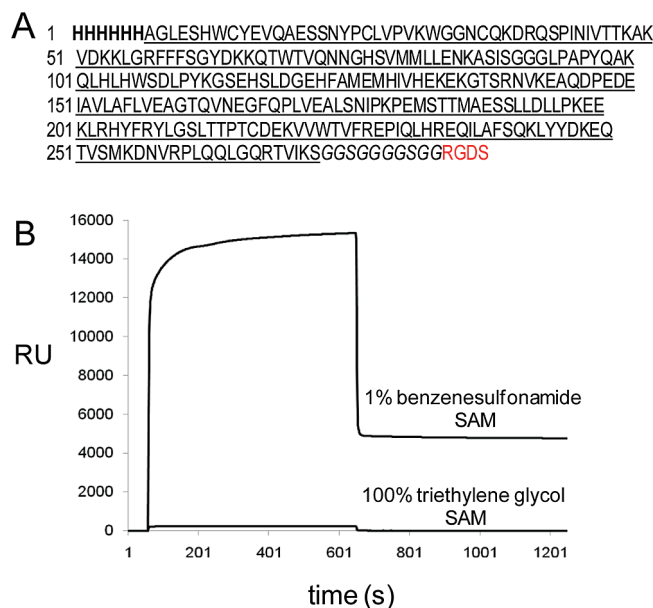
Figure 2. CA-RGD expression and binding to a benzenesulfonamide-presenting monolayer. (A) Primary structure of CA-RGD. His tag shown in bold, CAIV shown underlined, linker shown in italics, RGD domain shown in red. (B) SPR sensorgrams show that CA-RGD binds to 1% benzenesulfonamide SAMs but fails to bind to 100% tri(ethylene glycol) SAMs.
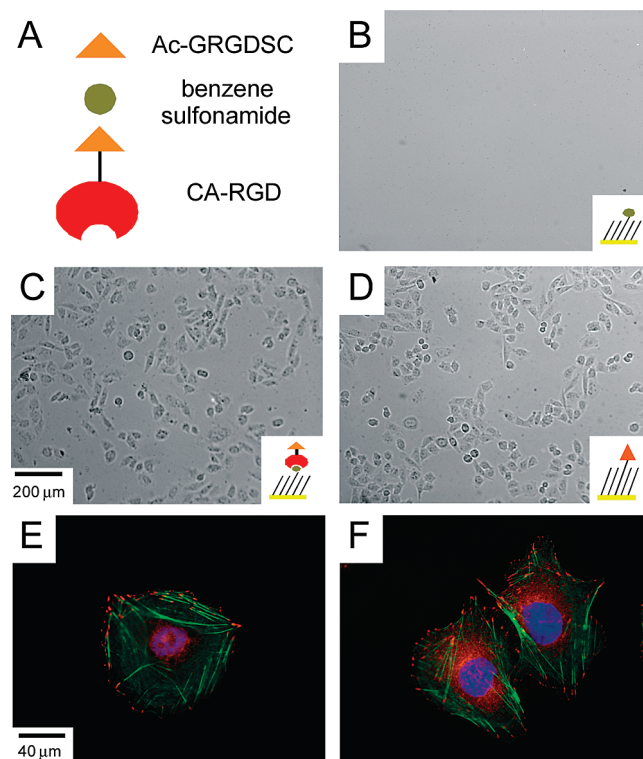
Figure 3. CHO cell adhesion and cytoskeletal structure mediated by CA-RGD on 1% benzenesulfonamide substrates (C,E) and 1% Ac-GRGDSC substrates (D,F). Cells failed to adhere to benzenesulfonamide monolayers (B).

ligand (Figure 3D). We fixed and stained the adherent cells with phalloidin to visualize the actin stress filaments and with an anti-vinculin antibody to visualize the focal adhesions. For both surfaces, cells had well-developed cytoskeletal structures (Figure 3E,F). Several control experiments establish the specificity of
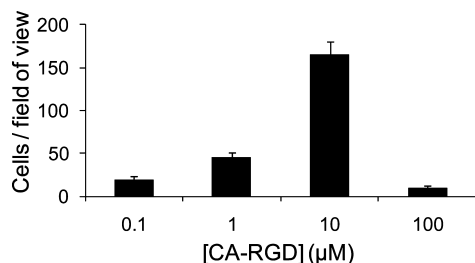
Figure 4. CA-RGD-mediated CHO cell adhesion depends on protein concentration. Error bars are the standard error of three experiments.

the ligand−receptor interaction in mediating adhesion. For example, CHO cells failed to adhere to monolayers presenting only tri(ethylene glycol) groups or the scrambled peptide Ac-GGRDGS (data not shown). Further, adhesion to the benzenesulfonamide-terminated monolayer in the presence of the adaptor protein could be inhibited by adding either bovine carbonic anhydrase or benzenesulfonamide ligand to the medium (data not shown).

CA-RGD-Mediated Cell Adhesion Is Concentration Dependent. The adaptor protein CA-RGD has a bivalent character, as it contains a domain that binds to cells and another that binds to the surface. Hence, the adaptor protein has the capacity to form a ternary complex with cells and the surface, or to bind to each independently. This dual character allows CA-RGD to either mediate adhesion or inhibit the adhesion of the cells. Because these roles should show a dependence on the concentration of protein, we compared the adhesion of CHO cells to monolayers presenting the benzenesulfonamide ligand with concentrations of the adaptor protein CA-RGD ranging from 0.1 to 100 μM (Figure 4). We found that the number of cells that attached increased with the concentration of the adaptor protein, reached a maximum at 10 μM, and then decreased with higher concentrations of protein. This trend is consistent with the bifunctional nature of the adaptor protein, since lower concentrations of protein should favor the equilibrium for binding of the protein to both the integrin and the benzenesulfonamide in a ternary complex, but higher protein concentration led to partitioning of the adaptor construct into binary complexes that would abrogate cell adhesion.

CA-RGD-Mediated Cell Adhesion Is Specific. As noted above, we found that both soluble RGD peptide and benzenesulfonamide ligand showed a dose-dependent inhibition of adaptor protein-mediated cell adhesion to the benezensulfonamide-terminated monolayer, with 50% inhibition occurring at inhibitor concentrations near 10 μM. Again, as evidence of the specificity of these interactions, the scrambled peptide GGRDGS had no effect on cell adhesion at any concentration tested (Figure 5A). We investigated the inhibition of adhesion by mixtures containing both the RGD peptide and the CA ligand, and we found that the mixture was more effective at blocking adhesion than was either ligand alone. Indeed, using mixtures that had different ratios of the two ligands—but always at a total concentration of 10 μM—we found that the strongest inhibition was observed when the ligands were present in a 1:1 ratio (Figure 5B).

Discussion

In this paper we report the use of an adaptor protein to restore cell adhesion to substrates that otherwise do not present ligands for the integrin receptors. We used a bifunctional protein that that can simultaneously bind to a benzenesulfonamide ligand
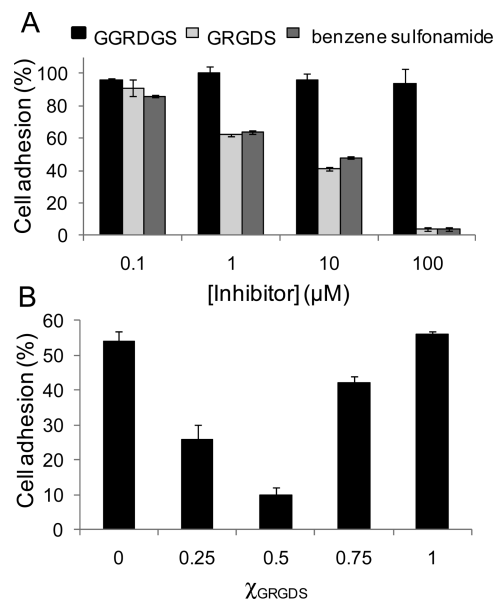
Figure 5. Characterization of CHO cell adhesion to CA-RGD on 1% benzenesulfonamide substrates. (A) Inhibition of cell adhesion in model system using soluble inhibitors. (B) Mixed inhibition of benzenesulfonamide and RGD peptide on CA-RGD-mediated cell adhesion. Error bars are the standard error of three experiments.

immobilized to a monolayer substrate and to a cell-surface integrin receptor on CHO cells. The bifunctional protein, CA-RGD, was effective in restoring cell adhesion to the monolayers and gave cell morphologies that were similar to those observed for cells adherent to an RGD-presenting monolayer. Hence, cells that were tethered to the surface by way of a noncovalently immobilized RGD ligand could still display normal cell adhesion and contractile cytoskeletal structures. The CA-RGD adaptor protein mediated specific adhesion, as shown by the ability of a soluble benzenesulfonamide ligand or RGD peptide to block adhesion. The most significant result is the demonstration that reversibly immobilized adhesion ligands can support the adhesion of cells.

The CA−benzenesulfonamide complex undergoes dissociation with a rate constant on the order of $1-10$ s^{-1}. However, the adhesion of cells engages a large number of CA−benzenesulfonamide complexes, and this polyvalent scaffold of interactions significantly increases the avidity of the complexes and consequently maintains a stable engagement of the cell with the surface. Mechanistic studies of polyvalent complexes have revealed them to be extraordinarily stable, often mediating binding events comparable to irreversibly bound ligands.[10,11] This stability derives from the improbability that each of the individual interactions is simultaneously in the dissociated state, which would be required for dissociation of the complex. Thus, although the CA−benzenesulfonamide interaction has a lifetime that is short relative to the time scale for cell adhesion, a threshold number of ligand−receptor bonds are maintained on average and able to support cell adhesion and spreading. Again, it is significant that the observed adhesion was comparable to that observed for substrates having a covalently immobilized RGD.

We note previous work that has used bimolecular complexes to immobilize adhesion ligands for promoting cell adhesion.

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For example, Reichert and co-workers immobilized a biotin-labeled RGD peptide to tissue culture plastic that was coated with a layer of streptavidin.[12,13] The resulting surfaces supported robust cell adhesion, but in this case the use of a streptavidin−biotin interaction provided for an essentially irreversible presentation of the RGD peptide and, in that sense, is more analogous to a covalent immobilization of ligands on a surface. By using a CA−benzenesulfonamide interaction to immobilize the RGD, we use an interaction that is rapidly reversible over the time scale of an adhesion experiment. We believe that this report is the first that localizes cell adhesion ligands to a surface in a reversible manner.

As discussed earlier, polyvalent systems combine two contradictory properties: they can associate to give an irreversible complex, but they can also be rapidly dissociated in the presence of a soluble ligand. We suggest that this property may enable a class of dynamic surfaces that can be used to change the identity of the immobilized ligand or remove the ligand completely and thereby release cells from the substrate. For example, by adding to the medium a fusion protein that binds the monolayer but that is modified with a second adhesion ligand, this protein is expected to exchange with the proteins that mediate adhesion and be driven by equilibrium to change the composition of adaptor protein at the cell−substrate interface. Similarly, a protein that can bind the substrate but that does not include an adhesion ligand may be useful for "removing" the RGD ligand and thereby releasing a cell culture without the need for enzyme treatment (as is now common and which can lead to unwanted proteolysis of cell−surface receptors).

Finally, we recognize the possibility of using this strategy to induce cell adhesion where the covalent introduction of cell adhesion ligands is not straightforward, particularly in *in vivo* settings. Many blistering diseases, for example, are associated with mutations that compromise the adhesion activity of extracellular matrix proteins.[14] Adaptor proteins that are designed to include a domain that binds to the mutated protein and a domain that binds to cells might represent a therapeutic solution for rescuing cell adhesion and stabilizing the tissue interface that is compromised in these diseases. This strategy is distinct from those under investigation that are based on protein injection and gene therapy[5,15] in that it involves an adaptor protein that would form a polyvalent scaffold at the site of injury and alleviate blistering symptoms, rather than the introduction of a native protein.

This report also illustrates the applicability of SAM substrates as mimics of the extracellular matrix.[2] The monolayers allow excellent control in orientations, densities, and compositions of immobilized ligands, whereas tri(ethylene glycol)-terminated monolayers are among the most effective at minimizing the nonspecific adsorption of protein. The combination of these benefits allows the molecular interactions between a cell and its substrate to be controlled. Finally, the monolayers can be directly characterized with MALDI-TOF MS and therefore can be structurally checked. In this work, we used SAMs to present the benzenesulfonamide moiety against an inert background, facilitating the specific recruitment of CA-RGD to the surface and the subsequent specific cell adhesion.

Conclusion

This work demonstrates the use of an exogenous protein to restore cell adhesion to a material that otherwise does not support adhesion. This engineering modality adds to the elegant strategies that have been demonstrated for biosynthetically modifying the cell surface or the surfaces of materials to introduce complementary interactions that mediate adhesion.[4,16,17] The present strategy is significant in that it does not require either the introduction of receptors in the cell or the modification of substrate with cell adhesion ligands, and therefore it may be applicable for creating surfaces that can be dynamically remodeled with different adhesion motifs and for repairing the loss of cell adhesion to mutated proteins *in vivo*. In the analogous use of soluble ligands to inhibit the adhesion of cells, for example, the monomeric ligands are far less effective than are corresponding multivalent ligands.[18] In this sense, the present work demonstrates a strategy for the self-assembly of a polyvalent scaffold from monomeric building blocks and suggests that this strategy may find broader use in the design and application of polyvalent ligands.

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