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Integrin Interactions with Immobilized Peptides in Polyethylene Glycol Diacrylate Hydrogels

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ABSTRACT

This study employs tissue-engineering technologies to evaluate neutrophil interactions with extracellular matrix (ECM)-mimetic peptides. We have used a polyethylene glycol (PEG) diacrylate derivative to form a hydrogel as a biologically inert surface. Covalent attachment of bioactive moieties to the hydrogel makes it bioactive. The goal is to define the mechanisms by which these moieties influence the interactions of neutrophils with this bioactive hydrogel, and thus understand the likely effects of similar ligands in the ECM. The current experiments analyze the interactions of isolated human neutrophils with PEG hydrogels modified with Arg-Gly-Asp-Ser (RGDS), a known ligand for some β_1 and β_3 integrins, and Thr-Met-Lys-Ile-Ile-Pro-Phe-Asn-Arg-Leu-Thr-Ile-Gly-Gly (TMKIIPFNRLTIGG), a ligand for Mac-1, a β_2 integrin. Our results demonstrate that neutrophils, independent of chemotactic stimulation, show little ability to adhere to unmodified PEG hydrogels. However, cell adhesion and spreading are robust on peptide-modified hydrogels. Incorporating distinct bioactive peptides, either alone or in combination, has enabled recognition of differential functions of $\alpha_v\beta_3$, β_1, and β_2 integrins on neutrophil adhesion and spreading. Combined interactions result in activity that differs markedly from that seen with either integrin independently engaged. This model allows investigation of specific ligand-induced leukocyte functions and the development of engineered matrices with defined bioactive properties.

INTRODUCTION

THE DEVELOPMENT of polyethylene glycol (PEG)-based hydrogels for use in both clinical and biological research applications has received much attention because of their excellent biocompatibility, including resistance to protein adsorption and cell attachment.[1–3] Some types of hydrogels can be modified by incorporating specific peptide sequences to promote cell–biomaterial interactions, serving as a valuable tool for investigating cellular dynamics.[1,2,4] Immobilization of biologically active peptide sequences, such as the RGDS sequence, can cause the normally inert and nonadhesive PEG-based hydrogel to become a bioactive material that supports robust cell adhesion.[1,2,4,5]

The RGD sequence is a ubiquitous adhesive peptide, responsible for the integrin–ligand interaction between many cells and multiple extracellular matrix (ECM) proteins, including fibrinogen, fibronectin, and vitronectin. Hern and Hubbell were among the first to incorporate the RGD peptide into a PEG diacrylate matrix, forming a hydrogel that induced specific receptor–ligand interactions,

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causing cell adhesion and cell spreading.[2] Further verification that PEG-immobilized peptides retain biological activity was demonstrated by Mann *et al.*, who modified the hydrogel to include adhesive and degradable peptide sequences, resulting in cell adhesion, cell spreading, and proteolytic degradation of the biomaterial.[4] Similar PEG-based biohybrid materials are employed here to investigate specific interactions between various ECM-derived peptides and neutrophils.

Cell adhesion, in particular, is often a necessary first step in basic cellular processes such as cell proliferation, motility, differentiation, apoptosis, cellular trafficking, and tissue development. In the case of neutrophils, adhesion events leading to motility and cellular trafficking are key to the function of these cells in wound healing and inflammation, particularly of concern in tissue injury. In chemokinetic signaling, integrins on the neutrophil surface are upregulated and activated. The cell is then primed for transmigration through the endothelial cell layer, and ultimately, directional migration through the ECM toward an inflammatory stimulus.

MATERIALS AND METHODS

Peptides

The peptide sequences used for adhesion and morphological studies were RGDS, TMKIIPFNRLTIGG, and YIGSR. RGDS, RGES (negative control peptide), and YIGSR peptides were purchased from American Peptide (Sunnyvale, CA). The TMKIIPFNRLTIGG sequence was synthesized with an automated peptide synthesizer (model 431A; Applied Biosystems, Foster City, CA) and standard 9-fluorenylmethoxycarboxyl (Fmoc) chemistry.

Development of biomimetic hydrogels

The biomimetic (PEG) hydrogels were prepared as previously described.[4,5] Briefly, PEG diacrylate was prepared by combining dry PEG (10,000 Da; 0.1 mmol/mL), acryloyl chloride (0.4 mmol/mL), and triethylamine (0.2 mmol/mL) in anhydrous dichloromethane and stored under argon overnight. The resulting PEG diacrylate was then precipitated with ether, filtered, and dried. PEG diacrylate was dialyzed before use. The polymer was analyzed by proton nuclear magnetic resonance (NMR) (Avance 400 MHz; Bruker, Billerica, MA).

Peptides were conjugated to PEG monoacrylate by reacting the peptide with acryloyl–PEG–*N*-hydroxysuccinimide (acryloyl–PEG–NHS, 3400 Da; Nektar Therapeutics, San Carlos, CA) in sodium bicarbonate (pH 8.5) at a 1:1 molar ratio for 2 h. The coupled acryloyl–PEG–peptide was lyophilized. Conjugated PEG–peptide was dialyzed before use. Materials in solvent dimethylformamide (DMF), including PEG and PEG–peptide copolymers, were characterized by gel-permeation chromatography and evaporative light-scattering detectors using polyethylene glycol standards (Polymer Laboratories, Amherst, MA).

Hydrogels were prepared by combining PEG diacrylate (0.1 g/mL) and acryloyl–PEG–RGDS (2.6–6.5 μmol/mL) or acryloyl–PEG–TMKIIPFNRLTIGG (2.0–4.9 μmol/mL) at 0.01–0.025 g/mL in phosphate-buffered saline (PBS, pH 7.2) containing glucose (0.1 g/L). The solution was then sterilized by filtration (pore size, 0.2 μm with 0.8-μm pore size prefilter; Pall Life Sciences, Ann Arbor, MI). 2,2-Dimethyl-2-phenyl-acetophenone (10 μL/mL) in *n*-vinylpyrrolidone (600 mg/mL) was added as the photoinitiator. The resulting solution was exposed to ultraviolet (UV) light (365 nm, 10 mW/cm^2) for 30 s to convert the liquid polymer solution to a hydrogel. The polymerized gels were then incubated overnight in PBS to allow them to reach their equilibrium swelling. PEG diacrylate hydrogels without peptides or with nonadhesive RGES (2.6–6.5 μmol/mL) or YIGSR (2.5 μmol/mL) peptide were used as controls.

Endotoxin detection

Endotoxin detection assays were conducted on polymer solutions before gelation. The *Limulus* amoebocyte lysate (LAL) assay was used to determine endotoxin presence in each component of the PEG hydrogel. Briefly, in sterilized glass tubes, 100 μL of sample was placed in a tube with 100 μL of LAL. The solution was placed on a heating block for 1 h and the end-point measure of gelation was observed. Endotoxin-negative solutions result in no gelation. Endotoxin at a concentration of 100 pg/mL will cause a sample solution to gelate. The negative control was pyrogen-free water (Baxter Healthcare, Deerfield, IL). The endotoxin-positive control was bacterial lipopolysaccharide (LPS). All samples of PEG hydrogel reagents were found to be endotoxin negative.

Neutrophil isolation

Neutrophils were obtained from the whole blood of healthy human volunteers. The method for isolation has been previously described.[6] Briefly, neutrophils were purified by 6% dextran sedimentation to remove red blood cells and Ficoll-Hypaque gradient centrifugation was applied to remove remaining leukocytes. The cells were finally suspended in phosphate-buffered saline containing glucose (0.1 g/L), to be used at a concentration of 1×10^6 cells/mL.

Reagents and antibodies

Neutrophil-stimulating agent, formyl-Met-Leu-Phe (fMLF), was obtained from Sigma (St. Louis, MO) and used at a concentration of 1×10^{-7} M. Mouse anti-hu-

man Mac-1 antibody 60.1 was received as a generous gift from L. Whitehouse (Repligen, Cambridge, MA) and used at a concentration of 10 μg/mL.[6] Anti-β_2 antibody R15.7 and mouse anti-human LFA-1 antibody R3.1 were provided by R. Rothlein (Boehringer Ingleheim, Ridgefield, CT) and each was used at a concentration of 10 μg/mL.[6] The mouse anti-human β_1 integrin monoclonal antibody JB1A was purchased from Chemicon International (Temecula, CA) and was used at a concentration of 20 μg/mL.[7] Mouse anti-human $\alpha_v\beta_3$ integrin monoclonal antibody LM609 was purchased from Chemicon International and was used at a concentration of 10 μg/mL.[8]

Static adhesion

Adhesion was quantified by a static adhesion assay previously described.[6,9] Briefly, biomimetic hydrogels were washed in protein-buffered saline and placed on the surface of a polystyrene cell culture dish. Neutrophils were seeded on the upper surface of the hydrogel and allowed to settle and adhere for 500 s. The 500-s time point is typically used for cells in the static adhesion chamber assays and is sufficient time for integrin-mediated cell adhesion to occur. Longer time points are being investigated in the context of cell motility experiments.[10] Once settled on the surface of the gel, the neutrophils were counted. The hydrogel was then inverted and the cells were allowed to fall away from the surface of the gel. Adherent neutrophils remaining on the hydrogel surface were counted; the fraction of adherent cells was calculated.

Cell spreading

Videotape of neutrophil activity was taken during static adhesion experiments described above. Each videotape was analyzed and frames were digitized with Optimas version 6.2 (MediaCybernetics, Silver Spring, MD). Neutrophils were randomly selected in various viewing fields ($n = 4$ fields per sample, ~30 cells per field). The analyzed frame dimensions were calibrated with a micrometer. Using the Optimas area morphometry data collection set, the projected surface area for each selected cell was calculated.

Statistical analysis

Data are expressed as means ± SE. All statistical significance was verified by analysis of variance (ANOVA) calculations (minimum, $n = 6$). Statistical significance is indicated by asterisks, where $p < 0.05$.

RESULTS

Adhesion of neutrophils to PEG hydrogels was evaluated under static conditions in a closed chamber without fluid motion, where the only force to remove the cells from the surface was the weight of the cell when the chamber was inverted. Under these conditions, approximately 20% of the cells remained attached to the hydrogel. Small amounts of shear applied to these cells reduced levels of neutrophil adhesion to <5%, supporting previous studies showing that PEG hydrogels are poorly adhesive for cells. When seeded on hydrogels containing the RGDS peptide, neutrophils adhered at levels significantly higher than those seen when the PEG hydrogels did not contain the adhesive peptide (Fig. 1A). With increasing concentrations of RGDS in the hydrogel, we saw increasing levels of unstimulated neutrophil adhesion (Fig. 1B). When stimulated with fMLF, a chemotactic tripeptide that activates neutrophils, twice as many adherent neutrophils were observed on the hydrogels containing RGDS at 2.6 μmol/mL (Fig. 1A). These results clearly indicate that a PEG hydrogel alone, without the inclusion of bioactive peptide sequences, allowed low levels of neutrophil adhesion. This is a significant result, as stimulated neutrophil adhesion is difficult to control without the use of antibodies or other integrin-blocking agents. These results support findings that suggest that polyethylene glycol surfaces are protein resistant, and support low levels of cell adhesion.[11] It is also clear that inclusion of the RGDS peptide, at various concentrations, promotes neutrophil adhesion.

The peptide sequence Arg-Gly-Glu-Ser (RGES) is commonly used as a control for experiments with RGDS. The RGES peptide has no known biological function. As seen in Fig. 1C, after seeding neutrophils on RGES-containing hydrogels, we saw no increase in cellular adhesion, whether or not cells were stimulated with fMLF. Adhesion did not increase on gels containing increasing concentrations of RGES. The low level of adhesion measured on PEG–RGES was the same as measured when neutrophils were seeded on inert PEG gels. These results indicate that, in contrast to RGDS, RGES does not provide a ligand for neutrophil adhesion and that the phenomena observed on hydrogels containing RGDS peptides were due to biospecific interactions.

Role of β_2 integrins in adhesion to bioactive peptide-containing hydrogels

Because the β_2 integrins are involved in the early neutrophil interactions of inflammation, it was important to investigate their involvement in adhesion to RGDS. The biomimetic hydrogel was used here to evaluate the isolated β_2 integrin dependence of neutrophils adhering to RGDS. Neutrophils were incubated with antibody R15.7, an antibody that binds and blocks the common β chain of all β_2 integrins.[6] Whether stimulated with fMLF or not, neutrophil adhesion to RGDS was not affected by incubation with this β_2 integrin-blocking antibody (Fig.

2A). In additional experiments, neutrophil inhibitory factor (NIF) was added to block Mac-1. NIF also failed to inhibit adhesion to RGDS-containing hydrogels (Fig. 2A). These results indicate that neutrophil adhesion to RGDS-containing hydrogels is not β_2 integrin dependent.

The TMKIIPFNRLTIGG peptide sequence has been shown to be a Mac-1-specific adhesion sequence on the γ chain of fibrinogen.[12] Mac-1 is a β_2 integrin involved in the initial capture and slow rolling of neutrophils during inflammation. Our use of the peptide sequence from the fibrinogen γ chain was supported by the experiments of Wright *et al*., who indicated that fibrinogen γ chain supports Mac-1-dependent binding of neutrophils to fibrinogen.[13] On immobilization of this peptide within the PEG-based hydrogels, unstimulated neutrophils were able to adhere at levels similar to those observed on RGDS-containing hydrogels (Fig. 2B). When stimulated with fMLF, there was a significant increase in the adhesion of

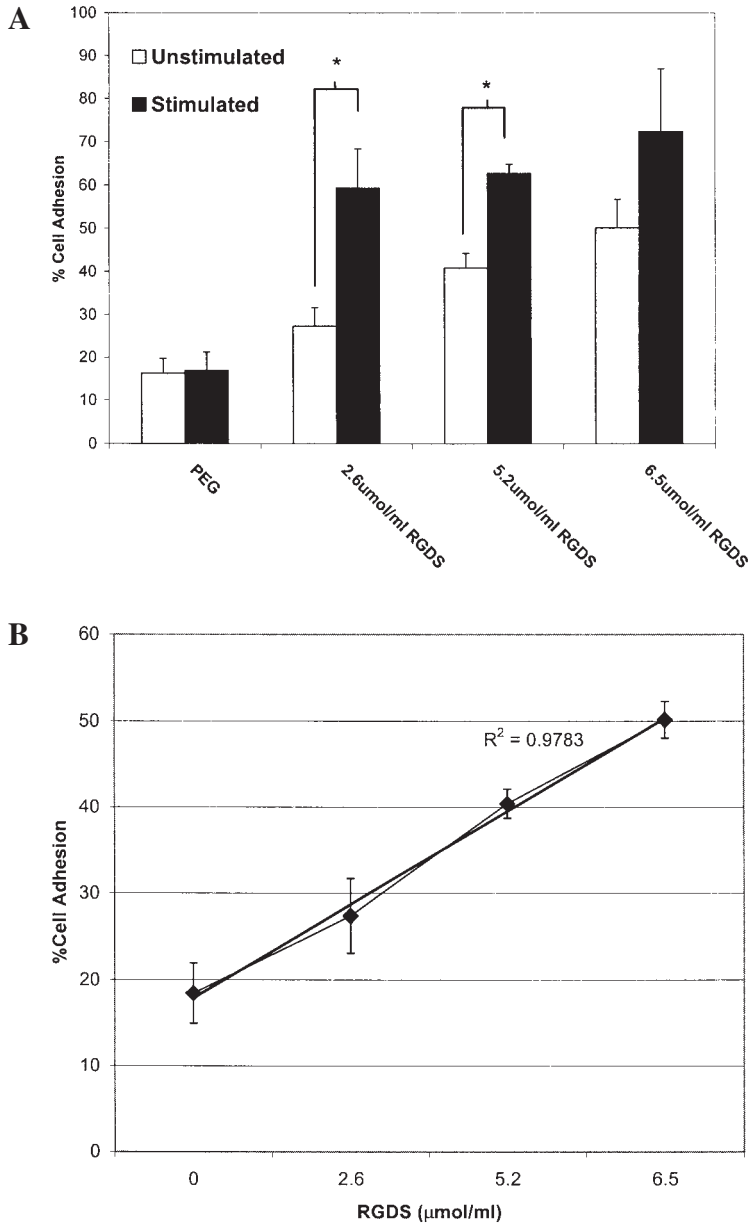


FIG. 1. Neutrophil adhesion on bioactive PEG–peptide hybrid. (**A**) The bioactive peptide sequence RGDS rendered the inert PEG bioactive by providing an adhesive ligand for integrin binding. There was significant adhesion to gels containing RGDS when cells were either unstimulated or stimulated with fMLF, although on chemokinetic stimulation cell adhesion was greatly enhanced. (**B**) Unstimulated neutrophil adhesion to RGDS-containing PEG hydrogels increases with increasing RGDS concentration. (**C**) There was no increase in neutrophil adhesion to hydrogels that contained the RGES peptide sequence. This indicates that adhesion to RGDS was not the result of nonspecific adhesion to peptides grafted onto PEG.

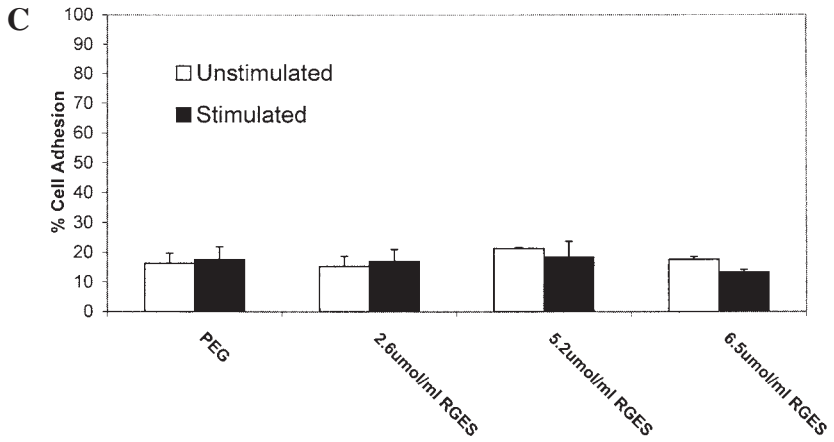


FIG. 1. *Continued.*

neutrophils to the TMKIIPFNRLTIGG-containing hydrogel, again similar to behavior on RGDS-containing hydrogels. Adhesion of neutrophils was dramatically reduced by treatment with the R15.7 antibody, indicating the adhesion was predominantly mediated by β_2 integrins. To distinguish which β_2 integrin is responsible for neutrophil adhesion to the TMKIIPFNRLTIGG peptide we attempted to inhibit adhesion with the LFA-1-blocking antibody R3.1,[6] and with the Mac-1 antibody 60.1.[6] Results are shown in Fig. 2C. After stimulating the neutrophils with fMLF and incubating them with each antibody, there was significant blocking of adhesion when neutrophils were incubated with 60.1, indicating a dominant role for Mac-1 in neutrophil adhesion to TMKIIPFNRLTIGG. R3.1 did not have a significant effect on neutrophil adhesion to this peptide, suggesting that LFA-1 is not involved.

Results of neutrophil adhesion to RGDS-containing hydrogels, using $\alpha_v\beta_3$ and β_1 integrin antibodies

As discussed above, neutrophil adhesion to the RGDS peptide was not dependent on β_2 integrins. Other integrins implicated in neutrophil adhesion during the inflammatory cascade are $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_4\beta_1$. Each of these integrins is thought to react with ECM proteins that contain the RGD sequence, including vitronectin, fibronectin, and fibrinogen.[14] To determine whether there was a specific interaction with one particular integrin, we attempted to block cell adhesion to RGDS-containing hydrogels with $\alpha_v\beta_3$ and β_1 integrin-blocking antibodies, with results shown in Fig. 3. Stimulated neutrophils were seeded on hydrogels modified with RGDS at 2.6 μmol/mL. These cells were incubated with either JB1A,[7] which blocks β_1 integrins, LM609,[8] which blocks $\alpha_v\beta_3$ integrin, or both antibodies. When neutrophils were incubated with JB1A alone, there was no significant decrease in cell adhesion. Treatment with LM609, however, did significantly reduce neutrophil adhesion to RGDS ($p < 0.05$). Interestingly, neutrophil adhesion to RGDS was more profoundly inhibited when cells were treated with JB1A and LM609 simultaneously. These results indicate that neutrophil adhesion to the RGDS peptide is primarily a function of $\alpha_v\beta_3$ integrin, with some contribution from β_1 integrins.

Roles of β_1 and $\alpha_v\beta_3$ integrins in neutrophil spreading on RGDS-containing hydrogels

As shown in Fig. 3, there is a role for β_1 integrins in adhesion to the RGDS peptide. Although they may not mediate firm adhesion to the bioactive peptide sequence, β_1 integrins may have a role in the morphological changes the cell undergoes once adherent to a substrate. To determine whether the β_1 integrins had any role in cell spreading, we analyzed digitized video images of neutrophils during the first 400 s of being seeded on hydrogels modified with RGDS at either 2.6 or 5.2 μmol/mL. The 400-s time point allowed enough time for the cells to settle on the surface and become firmly adherent. In Fig. 4, unstimulated neutrophils seeded on hydrogels containing RGDS at either 2.6 or 5.2 μmol/mL showed a small increase in surface area relative to cells seeded on PEG alone. These cells, although adherent, were round in shape, with a cross-sectional area measuring 94.6 ± 13.6 μm^2. When the neutrophils were stimulated with fMLF and seeded on RGDS (2.6 μmol/mL)-containing hydrogels, the surface area increased by more than 40% during the 400-s observation period, and on RGDS at 5.2 μmol/mL this increase was ~150%. In either case, when stimulated neutrophils were incubated with JB1A, the β_1 integrin-blocking antibody, cell spreading, but not attachment, was dramatically inhibited. These results indicate that, although β_1 integrins may not be necessary for adhesion to RGDS, they are necessary for cell spreading on RGDS.

Once we were able to determine an important role for β_1 integrins in neutrophil shape change, we chose to in-

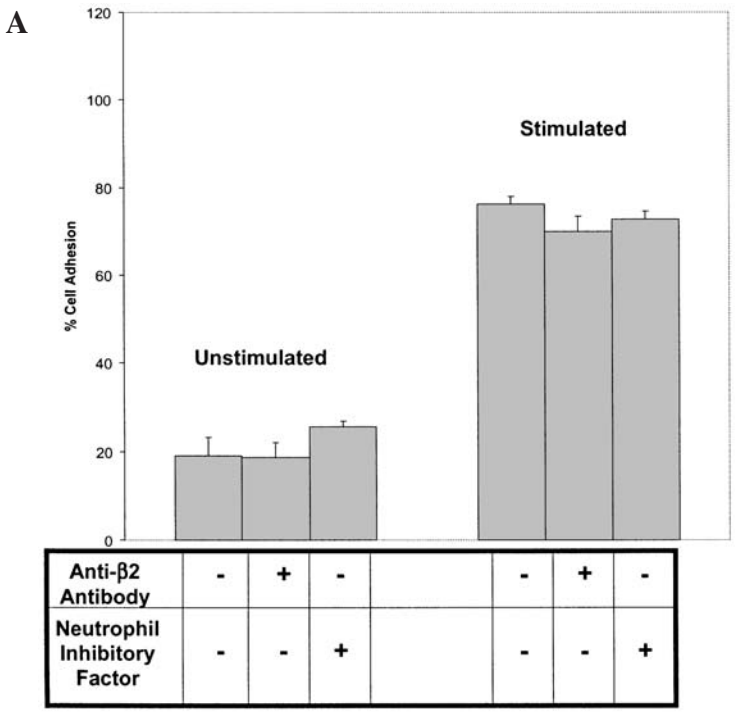


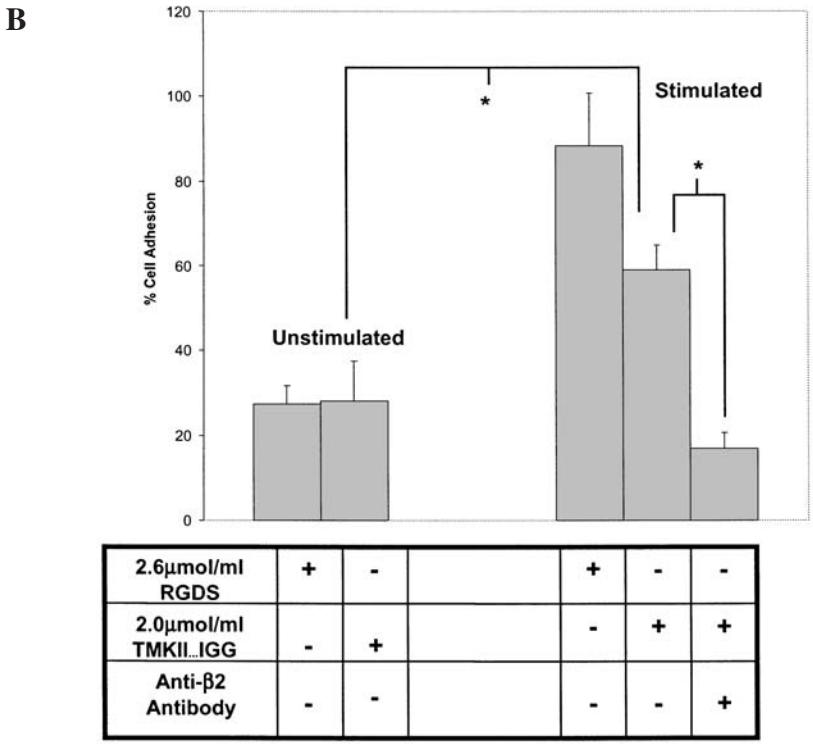
FIG. 2. β_2 integrin-independent adhesion to bioactive PEG–peptide hybrid. (**A**) Neutrophils seeded on PEG gels containing RGDS at 2.6 μmol/mL were adherent when stimulated with fMLF. This adhesion was not mediated by interactions between RGDS and β_2 integrins, as shown by the inability to inhibit adhesion via anti-β_2 integrin (R15.7) and β_2 integrin inhibitors (NIF). (**B**) Neutrophils seeded on PEG gels containing TMKIIPFNRLTIGG (2.0 μmol/mL) were adherent whether stimulated or unstimulated. Adhesion to this peptide was mediated by interactions between the peptide and β_2 integrins, shown by the ability to inhibit adhesion via R15.7, a β_2 integrin-blocking monoclonal antibody. (**C**) Neutrophils were seeded on hydrogels containing TMKIIPFNRLTIGG (2.0 μmol/mL). The cells were stimulated with fMLF and incubated with either LFA-1 antibody (R3.1), β_2 integrin antibody (R15.7), or Mac-1 antibody (60.1). Neutrophil adhesion to TMKIIPFNRLTIGG-containing hydrogels was significantly inhibited when using cells incubated with either R15.7 or 60.1.

vestigate the requirement for $\alpha_v\beta_3$ integrin in cell spreading. Digitized videotape was again analyzed in order to determine the increase in surface area when cells were seeded on hydrogels containing RGDS at either 2.6 or 5.2 μmol/mL. In these experiments, however, neutrophils were incubated with LM609, the $\alpha_v\beta_3$ integrin-blocking antibody, instead of JB1A. Here we saw that the increase in cell surface area, seen on gels containing RGDS at both 2.6 and 5.2 μmol/mL, was also blocked. Because in Fig. 3 we saw little adhesion to RGDS at 400 s when $\alpha_v\beta_3$ integrin was blocked, we did not expect to see spreading occur in cells during the initial contact period with the substrate. However, we needed to rule out possible transient shape change dependent on this integrin.

Similar studies were done with the R15.7 antibodies to assess the role of β_2 integrins in neutrophil spreading. For results shown in Fig. 4, the antibody used was the β_2 integrin-specific R15.7. When fMLF-stimulated neutrophils were incubated with the R15.7 antibody and seeded on either RGDS-containing surface, no significant effects on spreading were observed. These results indicate that β_2 integrins were not involved in the morphological changes the neutrophil experiences when firmly adherent to the RGDS sequence.

Results of neutrophil spreading on combined bioactive peptides

When hydrogels were modified with both RGDS and TMKIIPFNRLTIGG, substantial enhancements of neutrophil adhesion were observed (Fig. 5A). Our first observation was that neutrophils did not spread when induced to adhere to a surface via the Mac-1 integrin. Under stimulated and unstimulated conditions, there was no significant increase in cell surface area when neutrophils were seeded on the Mac-1-binding TMKIIPFNRLTIGG peptide. fMLF-stimulated neutrophils, seeded on PEG gels containing both RGDS and the Mac-1-binding fibrinogen peptide, TMKIIPFNRLTIGG, more than doubled in surface area. Surface area measurements of these cells were similar to those seen when neutrophils were seeded on PEG gels containing RGDS at 5.2



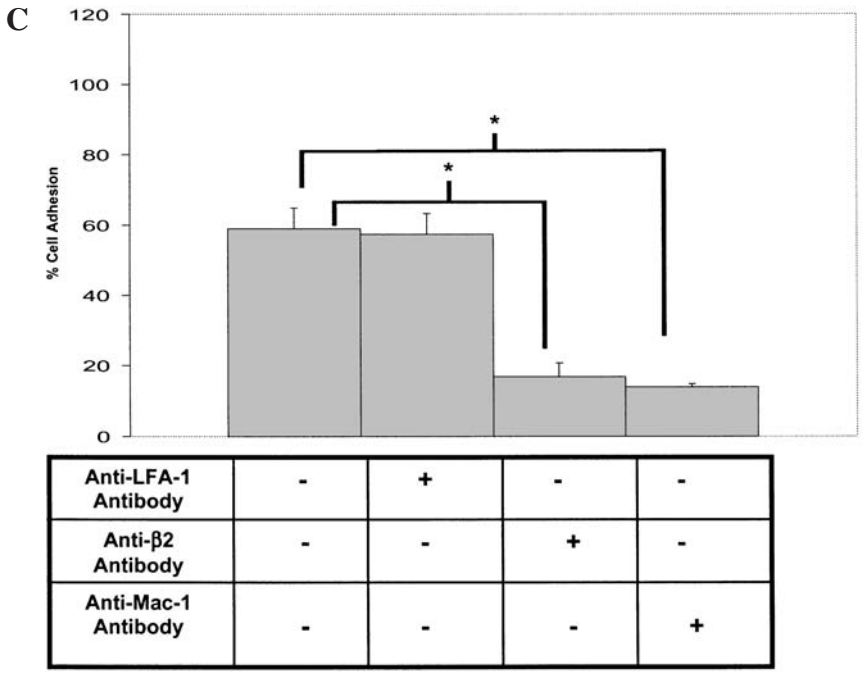


FIG. 2. *Continued.*

μmol/mL (Fig. 4). When the laminin nonintegrin peptide, YIGSR, was bound to the PEG chain, stimulated and unstimulated neutrophils did not show a significant increase in surface area relative to those seeded on gels containing the RGDS peptide alone. This was to be expected, as neutrophil spreading seems to be an integrin-mediated phenomenon and serves as an indication that spreading is mediated by specific ligand interactions.

In Fig. 5B, we evaluated the effect of inhibitors on cell spreading on gels containing a combination of RGDS (2.6 μmol/mL) and TMKIIPFNRLTIGG (2.0 μmol/mL) on the surface, and saw that stimulated neutrophils under-

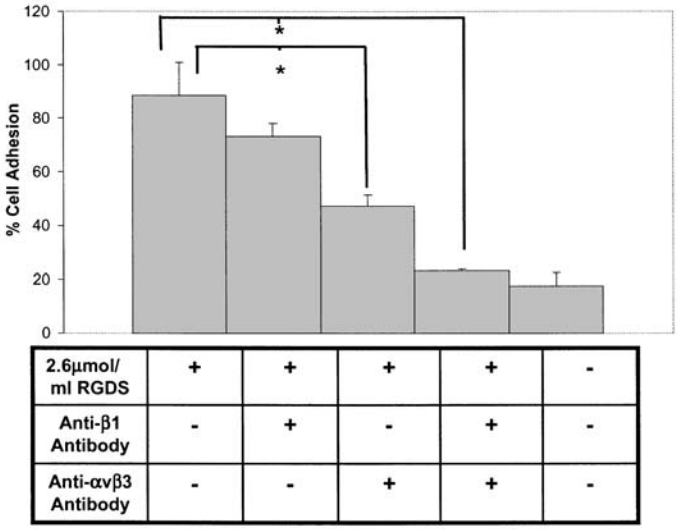


FIG. 3. Integrin-dependent adhesion to bioactive PEG–peptide hybrid. Adhesion to RGDS-containing PEG is partially inhibited by β_1 and $\alpha_v\beta_3$ integrin-blocking antibodies (JB1A and LM609, respectively). More dramatic inhibition was seen when neutrophils were incubated with the $\alpha_v\beta_3$ integrin-inhibiting antibody, suggesting that this integrin may be responsible for firm adhesion of neutrophils to RGD-containing protein.

went a dramatic increase in surface area. When incubated with LM609, JB1A, or 60.1, cell spreading was dramatically inhibited, indicating that each of the $\alpha_v\beta_3$, β_1, and Mac-1 integrins plays a role in cell spreading on the combined peptide hydrogel.

DISCUSSION

In this study we have shown that a polyethylene glycol hydrogel can serve as an inert surface to which there is little nonspecific neutrophil adhesion, providing a plat-

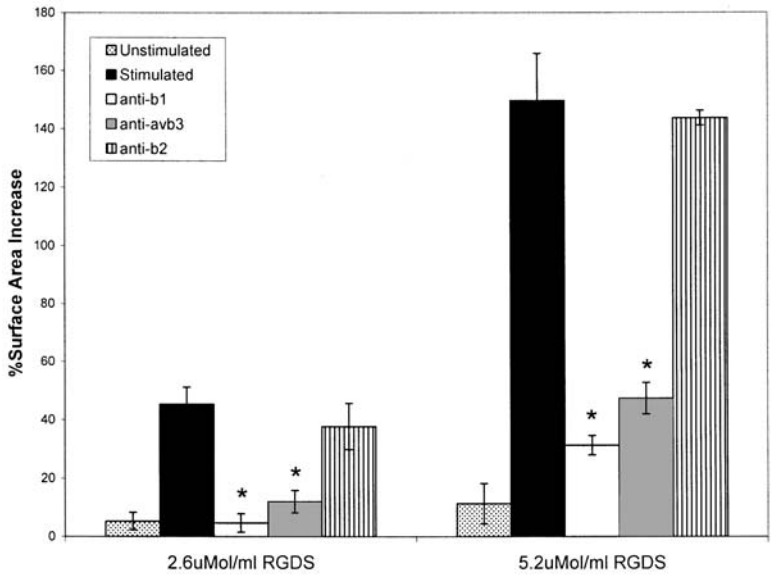


FIG. 4. Integrin-dependent neutrophil spreading on bioactive PEG–peptide hybrid. Neutrophils seeded on PEG containing RGDS exhibit shape change on adhesion. The measured surface area of stimulated cells more than doubled on the bioactive matrix. A more significant increase was seen when neutrophils were seeded on increasing concentrations of RGDS. The surface area increase of stimulated neutrophils was almost completely inhibited by blocking β_1 integrin. On inhibition of interaction between RGDS and the $\alpha_v\beta_3$ integrin, there was a dramatic inhibition in surface area increase. When incubated with R15.7, a β_2 integrin-blocking antibody, there was no effective blockage of cell spreading.

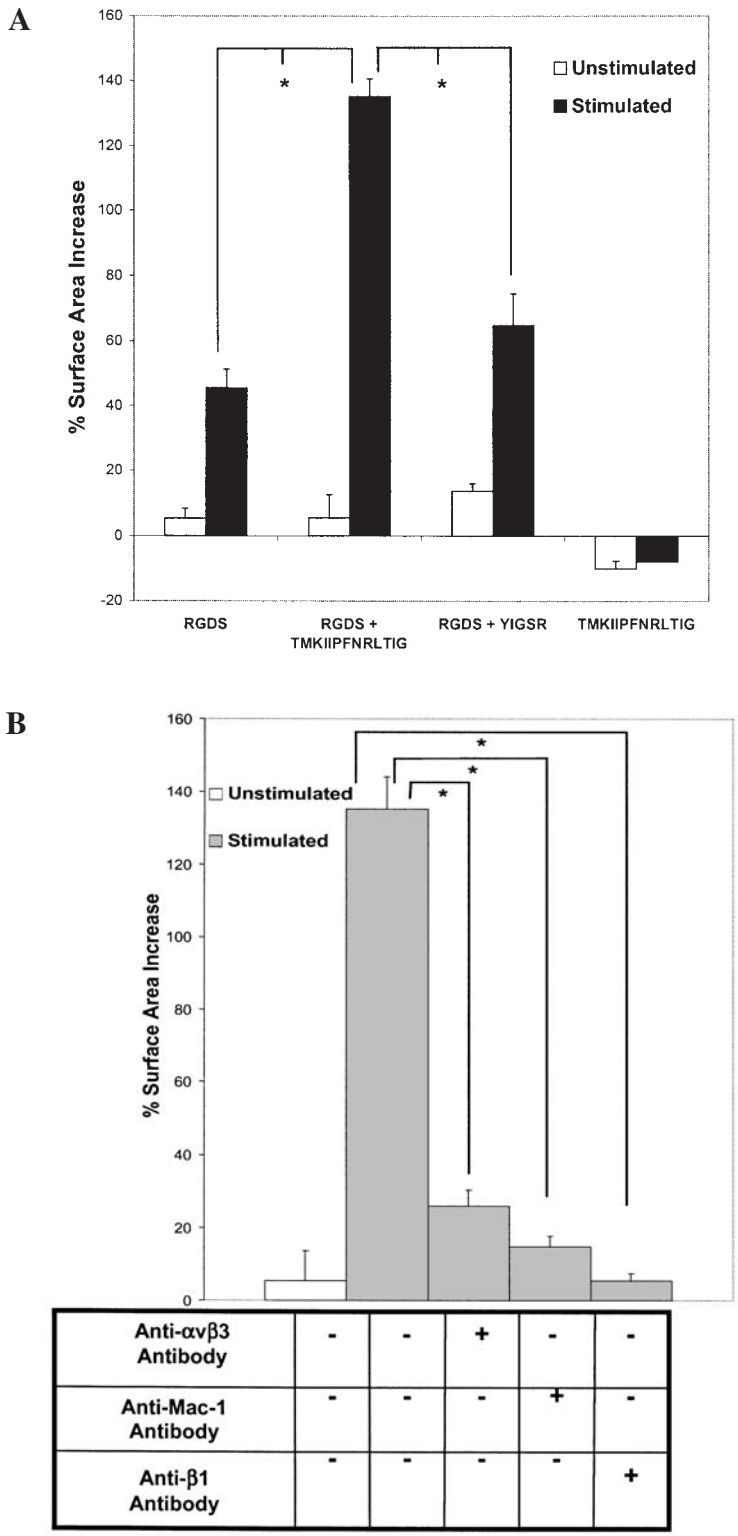


FIG. 5. Neutrophil spreading on bioactive PEG–peptide hybrid. (**A**) Stimulated and unstimulated neutrophils, seeded on PEG gels containing both RGDS and YIGSR, did not show a significant increase in surface area relative to those seeded on gels containing the RGDS peptide alone. Under stimulated and unstimulated conditions, there was no significant increase in cell surface area when neutrophils were seeded on the Mac-1-binding TMKIIPFNRLTIGG peptide. Stimulated neutrophils seeded on gels containing both RGDS and TMKIIPFNRLTIGG did show an increase in surface area that was significantly greater than that seen on RGDS alone. (**B**) Neutrophils seeded on hydrogels containing combined RGDS (2.6 μmol/mL) and TMKIIPFNRLTIGG (2.0 μmol/mL). Stimulated neutrophils experienced a dramatic surface area increase relative to unstimulated neutrophils on the same substrate. This increase was restricted when stimulated neutrophils were incubated with anti-$\alpha_v\beta_3$, anti-Mac-1, or anti-β_1 integrin antibodies.

form for assessment of specific effects of various adhesion ligands. Hern and Hubbell had previously shown that the PEG hydrogel could serve as a nonadhesive substrate when using human foreskin fibroblasts, and were also able to show that the inclusion of adhesive peptides could alter the characteristics of the material, rendering it bioactive.[2] The work here, however, extends to the neutrophil: inhibition of neutrophil adhesion to materials has been difficult. Similar studies were conducted, comparing neutrophil adhesion to biomaterials including glass, fluoroethylene propylene (FEP)-Teflon, polypropylene, and polyethylene. Consistently, under stimulated and unstimulated conditions, and under flow and static conditions, there was little neutrophil adhesion to the polyethylene surfaces at 10 min (<25% maximal adhesion on glass, stimulated and unstimulated), at which time significantly higher levels of adhesion were seen on all other experimental surfaces.[11] The resistivity of polyethylene to protein absorption may account for the decreased cell adhesion, in comparison with other biomaterials that more readily absorb protein to their surfaces.[15–17] This protein-resistant characteristic makes PEG an excellent candidate for an array of biomedical applications, especially those that involve direct blood contact. PEG may be considered an excellent polymer to coat biomaterial implants, helping to prevent protein adsorption and subsequent cell adhesion that may impede the function of the implant.

The current study has also shown that the PEG-based hydrogel can be used with the incorporated RGDS peptide sequence to create an extracellular matrix analog that mediates neutrophil adhesion. Neutrophil adhesion normally requires cell stimulation, but adhesion to these peptide-modified materials occurs in the absence of chemokinetic stimulation when the concentration of adhesive ligand is high enough. Using the bioactive hydrogel, we have been able to determine that neutrophil adhesion to RGDS involves both $\alpha_v\beta_3$ and β_1 integrins. Partial blockade of adhesion was seen when either integrin function was blocked with an antibody. Blocking the activity of both $\alpha_v\beta_3$ and β_1 integrins, simultaneously, resulted in complete inhibition of adhesion (Fig. 3). These results are consistent with reports that, in some melanoma lines, $\alpha_v\beta_3$ integrin is capable of recognizing the RGD sequence in fibronectin and vitronectin, resulting in $\alpha_v\beta_3$ integrin-dependent cell attachment.[18] A role for $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrin adhesion to RGD-containing proteins (fibronectin and fibrinogen) has also been suggested by a number of studies. Each suggests that engagement of β_1 integrins may result in cross-talk signaling of secondary integrins.[19–22]

The adhesive interactions between incorporated peptide sequences and neutrophil integrins result in enhanced cell spreading. This type of cellular shape change is commonly noted as an event that precedes cell migration.[23] When stimulated neutrophils were seeded on RGDS-containing hydrogels, there were morphological changes similar to those seen before degranulation[24] and cell movement.[22,25] Using blocking antibodies, we have experimentally determined that this cell spreading is primarily the result of β_1 integrin interaction with the RGDS adhesive peptide (Fig. 4). Previous studies had indicated that $\alpha_v\beta_3$ integrin adhesion to fibronectin acts in concert with $\alpha_5\beta_1$ integrin activation, resulting in firm attachment and cell spreading.[18] In combination, our results support the idea that $\alpha_v\beta_3$ integrin has a primary role in adhesion, whereas the β_1 integrins function as mediators of spreading. Because cell spreading has been related to neutrophil motility,[9,26] and secretory events,[24] a study of neutrophil protease and reactive oxygen species release on RGDS-containing matrices is currently underway. Stimulated neutrophil adhesion to fibrinogen and fibronectin can induce protease release.[24] The system developed here can be used to determine the specific integrin–peptide interactions that may lead to such degranulatory events.

We have shown that RGD-mediated adhesion of neutrophils is not β_2 integrin dependent (Fig. 2A). As β_2 integrins are constitutively present on the neutrophil surface and are commonly thought to be involved in firm adhesion of neutrophils,[13,14,27,28] it was interesting to discover that this important integrin is not involved in neutrophil interaction with the ubiquitous RGD sequence. Previous studies had indicated that there were interactions between the RGD sequence and Mac-1 integrin, a β_2 integrin. Studies showed that synthetic peptides containing RGD were able to block macrophages from binding to Mac-1 ligand-coated surfaces.[13,27–30] The Mac-1 adhesive ligands used included complement fragment C3bi,[27] fibrinogen,[13] filamentous hemagglutinin,[29] and glycoprotein 63.[30] Further reports suggested that when filamentous hemagglutinin underwent site-directed mutagenesis of its RGD sequence to RAD, the result was reduced binding of macrophages to filamentous hemagglutinin on bacteria, again suggesting a Mac-1-dependent adhesion to RGD.[29] However, after reviewing these findings and performing additional studies, Van Strijp *et al*. were unable to establish a direct effect of Mac-1 binding to RGDS.[27] Results shown here are consistent with those of Van Strijp and others, who showed that RGD peptides do not directly bind the adhesive sites of β_2 integrins.[31]

To induce β_2 integrin involvement in the neutrophil–matrix interaction, we used a Mac-1-specific sequence, isolated for its adhesive function.[12] Using the TMKIIPFNRTLIGG sequence, we were able to induce a Mac-1-specific adhesion interaction with the biomimetic hydrogel. Beyond showing that immobilized TMKIIPFNRTLIGG peptide retained its functionality, we showed that, alone, engagement of the Mac-1 integrin does not induce cell spreading. However, the TMKIIPFNRLTIGG peptide, in combination with RGDS, sup-

ported a neutrophil–peptide interaction that resulted in enhanced adhesion and spreading (Fig. 5A). This enhanced spreading was observed on materials modified with both peptides, suggesting a synergistic function of Mac-1 and RGD adhesive integrins. Using antibodies, we were able to determine that the spreading could be attributed to Mac-1, $\alpha_v\beta_3$, and β_1 integrins. van den Berg *et al*. observed similar synergistic activity of β_1 and β_2 integrins.[21] Using human neutrophils with fibrinogen- and fibronectin-coated microbeads, they obtained data suggesting that the engagement of β_1 integrins results in signaling that leads to Mac-1 activation and β_2 integrin-mediated firm adhesion. Imhof *et al*. have also presented data supporting cross-talk between $\alpha_v\beta_3$ and β_1 integrins, regulating cell migration.[32] Clearly, there are distinct but interconnected roles for each integrin and each is essential for normal neutrophil function.

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