Val-ala-pro-gly, an elastin-derived non-integrin ligand: Smooth muscle cell adhesion and specificity

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Abstract: The elastin-derived peptide val-ala-pro-gly (VAPG) may be useful as a biospecific cell adhesion ligand for smooth muscle cells. By grafting the peptide sequence into a hydrogel material, we were able to assess its effects on smooth muscle cell adhesion and spreading. These materials are photopolymerizable hydrogels based on acrylate-terminated derivatives of polyethylene glycol (PEG). Because of their high PEG content, these materials are highly resistant to protein adsorption and cell adhesion. However, PEG diacrylate derivatives can be mixed with adhesive peptide-modified PEG monoacrylate derivatives to facilitate cell adhesion. Following photopolymerization, PEG monoacrylate derivatives are grafted into the hydrogel network formed by the PEG diacrylate. This results in covalent immobilization of adhesive peptides to the hydrogel via a flexible linker chain. The resistance of PEG to protein adsorption makes it an ideal material for this model system since cell–material interactions are limited to biomolecules that are covalently incorporated into the material. In this case we were able to demonstrate that VAPG is specific for adhesion of smooth muscle cells. It also was shown that fibroblasts, endothelial cells, and platelets cannot adhere to VAPG. In addition, not only was smooth muscle cell adhesion dependent on ligand concentration, but also cell spreading increased with increasing ligand concentration. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 67A: 255–259, 2003

Key words: cell adhesion; elastin; smooth muscle cell; tissue engineering; vascular graft

INTRODUCTION

In the United States over 500,000 coronary artery bypass surgeries are performed each year.[1] In some cases natural tissues, such as autologous saphenous veins, can be used; however, suitable donor tissue often lacking is, and there are issues with donor site morbidity. Synthetic materials also are used, such as Dacron (polyethylene terephthalate, PET) and Gore-Tex (expanded polytetrafluoroethylene, ePTFE). These materials, however, have a lower patency rate than natural tissues, especially when used in small-diameter procedures (<6 mm). Tissue engineering may provide a technology for the development of new vessels for bypass procedures, particularly for small-diameter applications such as coronary artery bypass grafting (CABG).

To create a functional tissue-engineered vascular graft, it likely will prove necessary to recreate much of the native tissue structure. The arterial wall is composed of three distinct tissue layers. The innermost is the intima, which has a nonthrombogenic endothelial cell lining. The next layer is the media, which is comprised of smooth muscle cells (SMCs), elastin and laminin. The outermost layer is a connective tissue referred to as the adventitia. There are difficulties encountered in recreating this trilayered tissue structure because the cell types involved (endothelial cells, smooth muscle cells, and fibroblasts) proliferate and migrate at vastly different rates.

A means of ensuring development of the appropriate tissue structure may be to have biospecific and cell-selective adhesion within each of the layers. For example, Massia et al.[2] showed that the peptide sequence arg-glu-asp-val (REDV) is specific to endothelial cells and allows one to develop an intimal layer scaffold material that is preferentially adhesive to endothelial cells.

In normal vessels, the smooth muscle cells in the medial layer are surrounded primarily by and interact with elastin and laminin.[3] Val-ala-pro-gly (VAPG) is a peptide sequence derived from elastin.[4] This report will discuss the activity and specificity of VAPG for smooth muscle cell adhesion.

The soluble precursor of elastin is tropoelastin, which contains several hydrophobic repeating peptide sequences. The principal sequences are GGVP, PGVGV, and VGVAPG.[5] There is no evidence that

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elastin or tropoelastin interacts with integrins.[6] However, elastin sequences do bind to a non-integrin receptor, a galactoside-binding protein, with high affinity.[7]

This receptor is a multifunctional 67-kDa peripheral membrane protein found on smooth muscle cells and many other cells types.[7–10] The elastin binding protein recognizes several hydrophobic domains on elastin (VGVAPG, VGVPG, and VGAPG), laminin (LGTIPG), and type IV collagen (LGHVPG), which have similar secondary conformations.[7–9,11,12] However, binding occurs only in the absence of galactosugars.[7]

VAPG is derived from VGVAPG and serves as a quantitative marker for human elastins.[13] Though VGVAPG has been shown to assume a folded conformation that interacts with the hydrophobic regions of the elastin binding sequence,[8] the shortened sequence, VAPG, is found to have no preference for structured conformations.[14] Moreover, VAPG showed chemotactic and chemokinetic activity for monocytes similar to that of VGVAPG, but this activity was not inhibited by lactose.[14] Castiglione Morelli et al.[14] thus suggests that VAPG interacts with a receptor different from the elastin binding protein since chemotaxis is not reduced in the presence of lactose.

This report will demonstrate that VAPG can be used as a biospecific cell adhesion ligand for SMCs but not for platelets or fibroblasts. By grafting the peptide sequence into a cell nonadhesive hydrogel, we were able to assess its specific effects on cell adhesion and spreading. These materials are photopolymerizable hydrogels based on acrylate-terminated derivatives of polyethylene glycol (PEG). PEG diacrylate derivatives can be mixed with adhesive peptide-modified PEG monoacrylate derivatives.[15]

Following photopolymerization, the PEG monoacrylate derivatives are grafted into the hydrogel network formed by the PEG diacrylate.[15–17] This results in covalent immobilization of the adhesive peptides to the hydrogel via a flexible linker chain. The resistance of PEG to protein adsorption[18] makes it an ideal material for this model system since cell–material interactions are limited to the biomolecules that are incorporated covalently into the material. In this case we were able to demonstrate that VAPG is specific for smooth muscle cell adhesion.

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Synthesis of polyethylene glycol diacrylate

Polyethylene glycol (PEG) diacrylate was prepared by combining 0.1 mmol/mL dry PEG (6000 Da; Fluka, Milwaukee, WI), 0.4 mmol/mL of acryloyl chloride and 0.2 mmol/mL of triethylamine in anhydrous dichloromethane (DCM) under argon overnight. The resulting PEG diacrylate then was precipitated with ether, filtered, and dried *in vacuo*. The polymer was then dialyzed (MWCO 1000 Da) to remove any residual salts, lyophilized, and stored frozen under argon. The polymer was analyzed by proton NMR (Avance 400 MHz; Bruker, Billerica, MA; solvent: N, N-Dimethylformamide-d_7) to determine the degree of acrylation.

Synthesis of polyethylene glycol derivatives containing adhesive peptide sequences

The adhesive peptide sequences used were Val-ala-progly (VAPG; Sigma-Aldrich, St. Louis, MO), arg-gly-asp-ser (RGDS; Sigma-Aldrich, St. Louis, MO), and a scrambled sequence val-pro-ala-gly (VPAG), which was synthesized using standard dicyclohexylcarbodiimide (DCC) activation and Fmoc protection chemistry on an Applied BioSystems peptide synthesizer (Model 431A; Foster City, CA).

Peptides were conjugated to PEG monoacrylate by reacting the peptide with acryloyl-PEG-NHS (3400 DA; Shearwater Polymers, Huntsville, AL) in 50 m*M* of sodium bicarbonate (pH 8.5) at a 1:1 molar ratio for 2 h. The mixture then was dialyzed (MWCO 3500 Da), lyophilized, and stored frozen under argon. Gel permeation chromatography with UV-vis and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA) was used to determine the coupling efficiency.

Hydrogel photopolymerization

Hydrogels were prepared by combining 0.1 g/mL of PEG diacrylate derivative and 5.24 mg/mL (1.4 μmol/mL) of acryloyl-PEG-VAPG in 10 m*M* of HEPES buffered saline (pH 7.4, HBS). The solution then was sterilized using a syringe filter (25-mm Acrodisc, 0.8/0.2 μm of Supor® membrane; Pall Gelman Laboratories, Ann Arbor, MI). Ten μL/mL of 2,2-dimethyl-2-phenyl-acetophenone in n-vinylpyrrolidone (600 mg/mL) then were added as the long wavelength UV photoinitiator.

The polymer solution then was placed into glass molds and exposed to UV light (365 nm, 10 mW/cm^2) for 30 s to convert the liquid polymer to a hydrogel. Hydrogel samples were stamped out using a 6-mm diameter punch and placed into a 48-well plate with media overnight to allow the hydrogels to reach their equilibrium swelling. PEG diacrylate hydrogels without the adhesive VAPG or with the scrambled sequence VPAG component (5 μmol/mL) were prepared as controls.

Cell maintenance

Human dermal fibroblasts (HDFs; Clonetics, San Diego, CA), bovine aortic endothelial cells (BAECs; Clonetics, San Diego, CA), human aortic smooth muscle cells (HASMCs;

Cell Applications, San Diego, CA), and Sprague-Dawley smooth muscle cells (SDSMCs; isolated from the thoracic aorta of Sprague-Dawley rats and characterized, as previously described[19]) were maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 2 m*M* of L-glutamine, 500 U of penicillin, and 100 mg/L of streptomycin. Cells were incubated at 37°C in a 5% CO_2 environment.

Cell spreading and morphology

Hydrogel samples with varying concentrations of VAPG (0.0, 1.4, 2.8, 3.5, and 5.0 μmol/mL), or 5.0 μmol/mL of VPAG were allowed to swell in media to equilibrium overnight. The media was changed, and SDSMCs (passage 10, 20,000 cells/well) were added to the top surface of the hydrogels. For additional controls, HDFs (passage 7, 20,000 cells/well) and BAECs (passage 8, 20,000 cells/well) were seeded in a similar fashion on hydrogels containing 5.0 μmol/mL of VAPG.

The hydrogels then were incubated for an additional 20 h to allow cell attachment and spreading. After the incubation period, digital images of the hydrogel surfaces were taken with a CCD camera mounted on a phase contrast microscope (Axiovert 135; Carl Zeiss, Thornwood, NY). Cell spreading was evaluated using image processing to determine the area of individual cells on each surface (NIH Scion Image, version 1.62C; developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Platelet adhesion

Hydrogels containing VAPG (5 μmol/mL) and control collagen gels were incubated at 37°C for 15 min with 4 mL of whole blood containing heparin (10 U/mL) and mepacrine (10 μ*M*), which fluorescently labels platelets and white blood cells. The gels were gently rinsed 6–8 times with phosphate-buffered saline (PBS; Sigma). The gels then immediately were examined by taking digital images with a CCD camera mounted on a fluorescent microscope (Axiovert 135; Carl Zeiss, Thornwood, NY). The number of remaining adhered platelets per field of view was determined.

Identification of the VAPG receptor

PEGDA hydrogels containing VAPG (5 μmol/mL) and control PEGDA hydrogels containing RGDS (5 μmol/mL) were made and allowed to swell to equilibrium overnight in serum-free media. HASMCs (passage 9, 20,000 cells/mL) were trypsinized and then incubated in serum-free media with or without a β1 integrin subunit antibody (1 μg/mL; mouse monoclonal to human beta 1 integrin, ab7168; Abcam Limited, Cambridge, UK) for 15 min. The HASMCs then were seeded on hydrogel surfaces and incubated for 1 h.

Digital images of hydrogel surfaces were taken with a CCD camera mounted on a phase contrast microscope (Axiovert 135; Carl Zeiss, Thornwood, NY). Cell attachment and spreading was determined. Similar studies were performed to assess ligand binding to the β3 integrin subunit (mouse monoclonal to human integrin beta 3, MAB2023Z; Chemicon International, Temecula, CA).

In order to assess cell adhesion with the use of the elastin receptor, PEGDA hydrogels were made with either VAPG (5 μmol/mL) or VGVAPG (5 μmol/mL) and allowed to swell to equilibrium overnight in serum-free media. HASMCs (passage 9, 20,000 cells/mL) were incubated for 15 min in the presence of either VGVAPG (1 μg/mL; Sigma-Aldrich, St. Louis, MO) in serum-free media or just serum free media alone. The cells then were seeded on the hydrogel surfaces and allowed to attach and spread for 1 h, after which digital images were taken and analyzed as described above.

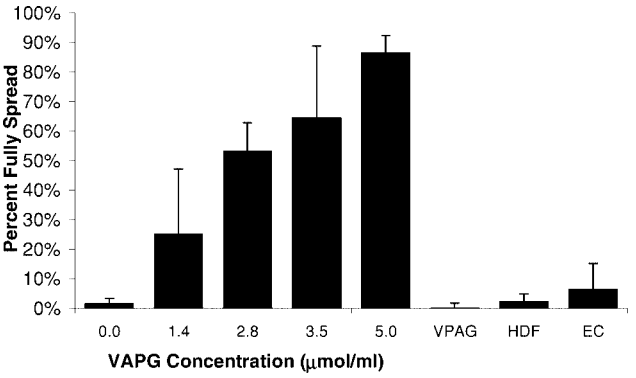

Figure 1. The percent of fully spread smooth muscle cells on hydrogel surfaces with varying concentrations of VAPG. A linear correlation was found between ligand concentration and number of attached and spread smooth muscle cells. Surfaces without VAPG, or with VPAG (5 μmol/mL) have no cell adhesion. Also, when HDFs or EC were seeded onto surfaces with 5.0 μmol/mL of VAPG, no adhesion was seen. Bars represent mean + standard deviation; $n = 3$.

Statistical analysis

Data sets were compared using two-tailed, unpaired *t* tests. *P* values of less than 0.05 were considered significant. The error bars in the figures represent standard deviations.

RESULTS

Cell adhesion and morphology

After a 20-h incubation period, cell adhesion and spreading on the surfaces of hydrogels with varying concentrations were analyzed. It was found that with increasing VAPG concentration, the number of at-

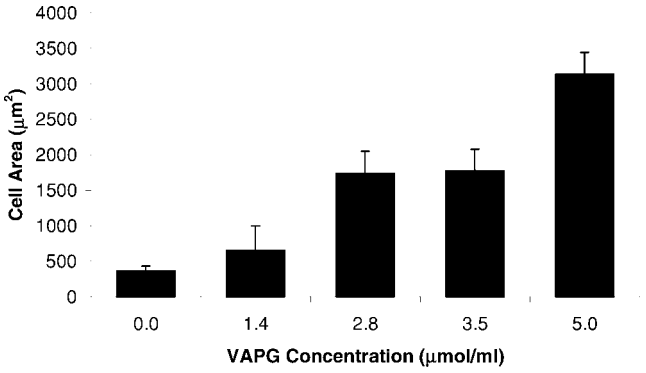

Figure 2. Smooth muscle cell area of attached cells to surfaces with varying concentrations of VAPG. Cells spread more with increasing concentrations of VAPG. Bars represent mean + standard deviation; $n = 3$.

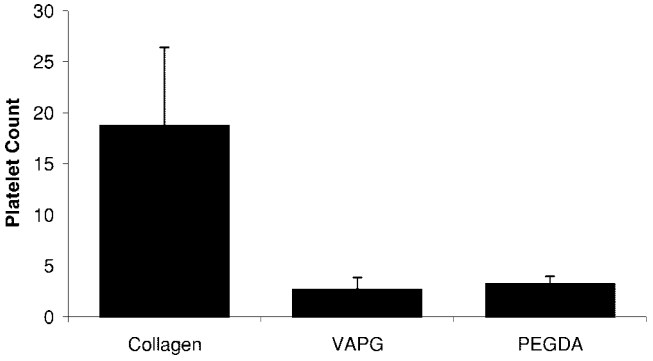

Figure 4. Count of platelets adhered to hydrogel surfaces. Platelets did not adhere to VAPG-modified hydrogels (VAPG: PEG diacrylate with 5.0 μmol/mL of VAPG) or on PEG diacrylate hydrogels (PEGDA). However, platelets did adhere to collagen gels. (Bars represent mean + standard deviation; $n = 10$.

tached and spread SMCs increased (Fig. 1). In addition a linear correlation was found between the ligand concentration and the number of spread cells ($r^2 = 0.9963$). The area of the spread smooth muscle cell was also calculated, and it was found that the cell area increased with increasing ligand concentration (Figs. 2 and 3). However, smooth muscle cells did not adhere to hydrogels with a high concentration of the scrambled peptide VPAG (5 μmol/mL). It is important that neither fibroblasts nor endothelial cells were able to adhere to hydrogels that contained VAPG (5 μmol/mL).

Platelet adhesion

Platelets did not adhere to hydrogels modified with VAPG, as compared to control collagen surfaces. In fact, platelet adhesion to VAPG-modified hydrogels was similar to that of platelet adhesion to nonadhesive PEGDA hydrogels ($p = 0.23$; Fig. 4).

VAPG receptor identification

In order to rule out specific receptors that may bind VAPG, blocking studies were performed. The elastin receptor (β1 or β3 integrin subunits) was chosen as the preliminary receptor for investigation of ligand binding. In a 1-h period, it was seen that HASMC adhesion did not significantly decrease in the presence of VGVAPG ($n = 6$, $p = 0.18$) or in the presence of the β1 integrin subunit antibody ($n = 6$, $p = 0.56$). However, it appears that β3 may be involved in binding to the VAPG ligand, and further studies will need to be conducted to confirm the data.

DISCUSSION AND CONCLUSIONS

Blood vessels are made up of three layers: intima, media, and adventitia. Each layer is composed of a specific cell type and provides different functions. The intimal layer is composed of a monolayer of endothe-

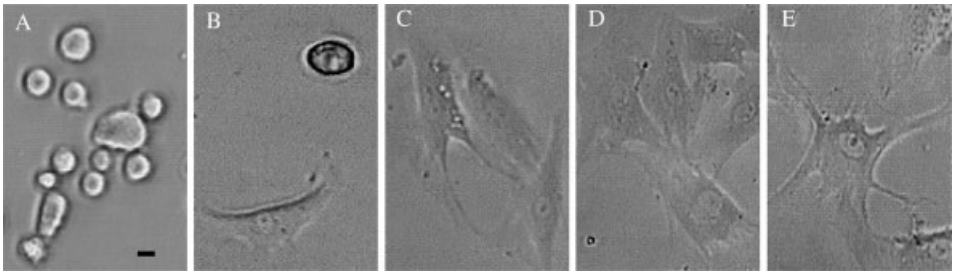

Figure 3. Micrographs of smooth muscle cells on hydrogel surfaces with varying concentrations of VAPG. Scale bar represents 10 μm. (A) Cells are rounded and not fully attached to surface (0.0 μmol/mL). (B) Cells are attached and begin to spread on surfaces with 1.4 μmol/mL of VAPG. (C,D) Cells are attached and very spread on surfaces with 2.8 μmol/mL and 3.5 μmol/mL, respectfully. (E) Cells are fully extended on surfaces with 5.0 μmol/mL of VAPG. Lamellae are extended in many directions.

lial cells that provide a nonthrombogenic surface and secrete substances that maintain smooth muscle phenotype and tone. The medial layer is comprised of smooth muscle cells and ECM proteins that provide the mechanical and contractile properties of the vessel. The adventitial layer is made up primarily of fibroblasts and incorporates connective tissue, capillaries, and nerves to the vessel.

In designing a tissue-engineered vascular graft, many groups have chosen to focus on only one layer of the vessel, the medial layer, to provide the mechanical stability of the graft.[20–23] However, to reduce thrombogenicity, an intimal layer composed of endothelial cells is required. Massia et al.[2] has shown that biomaterial surfaces can be developed with specificity for endothelial cell adhesion by grafting in an adhesion peptide sequence, arg-glu-asp-val (REDV), that interacts with a receptor found on endothelial cells but not on fibroblasts, smooth muscle cells, or platelets. Ideally, the medial layer should to be designed to provide similar specificity for smooth muscle cell adhesion. This can be accomplished by biomaterial modification with an adhesion ligand that is specific to smooth muscle cells.

The results of this study indicate that the peptide sequence VAPG is specific for smooth muscle attachment and adhesion. The number of fully spread smooth muscle cells increased with increasing concentration of VAPG. In addition, the spread-cell area increased with increasing ligand concentration. However, attachment was not observed when the scrambled peptide sequence, VPAG, was used. It also was observed that fibroblasts, endothelial cells, and platelets could not adhere to surfaces containing VAPG.

The specificity of this ligand is important to the development of tissue-engineered vascular grafts. Incorporation of VAPG that is preferential for smooth muscle cells in a scaffold will allow one to develop a medial layer, thus leading to a recreation of the trilayer structure of a natural vessel.

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