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Modification of surfaces with cell adhesion peptides alters extracellular matrix deposition

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Abstract

The goal of the current study was to evaluate matrix protein synthesis by cells cultured on materials that had been modified with cell adhesion ligands. We examined the effects of surface peptide density and of peptides with different affinities on the extracellular matrix production of smooth muscle cells, endothelial cells and fibroblasts. While initial adhesion was greatest on the higher density peptide surfaces, all cell types exhibited decreased matrix production on the more highly adhesive surfaces. Similarly, when different peptides were evaluated, matrix production was the lowest on the most adhesive surface and highest on the least adhesive surface. These results suggest that extracellular matrix synthesis may be regulated, to some extent, by signal transduction initiated by adhesion events. This may pose limitations for use of bioactive materials as tissue engineering scaffolds, as matrix production is an important aspect of tissue formation. However, it may be possible to increase matrix production on highly adhesive surfaces using exogenous factors. TGF-β was shown to increase matrix production by both smooth muscle cells and endothelial cells.

Keywords: ECM; TGF-β; Surface modification; SMC; Endothelium

1. Introduction

Bioactive materials offer a way to control cell–material interactions. To modify biomaterial surfaces, bioactive ligands such as peptides and polysaccharides may be either adsorbed or covalently grafted to the surface or included in the bulk composition. Cell adhesion peptides have been evaluated extensively for surface modification to enhance cell adhesion or to allow biospecific cell adhesion. In general, however, the effects of these peptides on aspects of cell behavior other than adhesion are not well understood. The tripeptide RGD sequence, found in many extracellular matrix proteins, is the binding motif for cell surface integrin receptors and has been extensively studied. RGD covalently bound to glass substrates has been found to increase adhesion and spreading of fibroblasts [1–3] and spreading of bovine pulmonary artery endothelial cells [4]. RGD grafted onto PHEMA and PET also increased fibroblast adhesion, although no stress fiber formation was seen, possibly indicating an incomplete adhesive response. In contrast, porcine aortic endothelial cells seeded on the same surfaces showed increased initial spreading as compared to untreated polymers, but by 24 h these differences were no longer evident [5]. Bovine aortic endothelial cells seeded upon RGD-modified PLLA showed increased spreading at 4 h when compared to the same cells on unmodified PLLA [6,7]. An RGD-containing peptide covalently bound at high density to a dialdehyde starch coating on Thermanox coverslips and on polystyrene plates enhanced adhesion, spreading and growth of human umbilical vein endothelial cells compared to their behavior on fibronectin-coated polystyrene [8].

YIGSR, an integrin receptor ligand derived from laminin, has also been studied extensively as an adhesion peptide. YIGSR covalently bound to glass substrates was also found to increase spreading and proliferation of bovine pulmonary artery cells [4], but studies with fibroblasts remain controversial with claims of both increased [1] and decreased [3] adhesion being reported.

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These differences may be due to differences in cell species used, in the surface peptide concentration (12 pmol/cm^2 vs. 10 nmol/cm^2), or the method of attaching the peptide (glycophase glass vs. aminophase glass). Glycophase glass is intrinsically non-adhesive while aminophase glass is intrinsically adhesive. REDV, a peptide derived from fibronectin, allows specific adhesion of endothelial cells and thus is promising for use in cardiovascular tissue engineering. When REDV was covalently bound to glycophase glass or to a PET surface modified with polyethylene glycol, endothelial cells attached and spread, but fibroblasts, smooth muscle cells and platelets did not [9].

Tissue engineering and wound healing are fields where bioactive materials with cell adhesion ligands are utilized. Biomaterials with specific ligands can potentially control and facilitate tissue formation or regeneration. However, in order to ensure proper development of the desired tissue, there needs to be complete understanding of cellular interactions with bioactive materials and their potential to impact tissue formation. One important aspect of tissue formation is production of extracellular matrix (ECM) components, as the composition of the ECM largely determines the properties of the tissue. The amount of matrix produced by cells growing on a scaffold is especially important as it could determine the viability of a tissue engineered product. In the current study, we address matrix protein production by fibroblasts, smooth muscle cells and endothelial cells as a function of bioactive ligand affinity and density. We hope to apply these findings to the development of novel scaffolds for tissue engineered vascular grafts.

2. Materials and methods

2.1. Surface preparation

Chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. Cell adhesion peptides used were RGDS, YIGSR, VAPG, VGVAPG and KQAGDV. The peptide RGES was used as a negative control. The peptides were acetylated by adding an equimolar amount of acetic anhydride to the peptide (equimolar to the number of amines on the peptide) and allowing them to react at room temperature for 2 h. The resulting acetylated peptide was then lyophilized. Aminophase glass slides were prepared by placing clean glass slides in dry acetone with 4% (v/v) 3-aminopropyltriethoxysilane and incubating at 37°C overnight. The slides were then rinsed in acetone and sonicated in deionized water for 15 min. To couple the peptides to the aminophase glass, the slides were placed in *N*,*N*-dimethylformamide (DMF) containing 1.5 mg/ml 1-ethyl-3-(3-diethylaminopropyl) carbodiimide, 2% (v/v) *N*-ethylmorpholine, and acetylated peptide at 5 or 50 nmol/ml. The slides were then incubated at 37°C for 4 h [3,10]. The coupled slides were rinsed in DMF and then deionized water, sonicated in 4 M urea for 10 min, sonicated in 1 M NaCl for 10 min, and rinsed with deionized water. Aminophase and peptide-coupled slides were assayed for amine content on the surface using the ninhydrin assay and comparing to results obtained with leucine standards (12.5–100 μM) [11]. Slides were sterilized under UV light overnight prior to use.

2.2. Cell maintenance

Cell types used in this study were: smooth muscle cells from the thoracic aorta of spontaneously hypertensive rats (SHR), bovine pulmonary artery endothelial cells (BAEC), human aortic smooth muscle cells (HASMC; Clonetics, San Diego, CA), human umbilical vein endothelial cells (HUVEC; Clonetics), and human dermal fibroblasts (HDF; Clonetics). SHR and BAEC were isolated and characterized as previously described [12,13]. SHR and HASMC were maintained on Minimal Essential Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 500 units penicillin and 100 mg/l streptomycin. BAEC, HUVEC and HDF were maintained on Dulbecco's Modified Eagle's Medium with low glucose (1 g/l) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 500 units penicillin and 100 mg/l streptomycin. Cells were incubated at 37°C in a 5% CO_2 environment.

2.3. Evaluation of matrix protein production by cultured cells

Cell suspensions were prepared in their appropriate media supplemented with 5 μg/ml ascorbic acid at a concentration of 10 000 cells/ml for the peptide density studies and 40 000 cells/ml for the remaining studies. Cell suspensions to be used for measurement of ECM protein production were also supplemented with 1 μCi/ml ^{3}H-glycine (40 Ci/mmol). The glass slides, prepared as described above, were attached to FlexiPerm strips in QuadriPerm Cell Culture Vessels (Heraeus, Osterode am Harz, Germany) to create eight wells ($1.11 \times 0.79 \times 0.79$ cm) on each slide. Four of the wells on each slide were utilized to measure the ECM production while the remaining four wells were utilized for cell number determination and were cultured in the absence of ^{3}H-glycine. Assays of cell numbers were performed at the same time as matrix production assays described below by preparation of single cell suspensions using trypsin and counting samples using a Coulter counter (Multisizer #0646, Coulter Electronics, Hialeah, FL).

To evaluate synthesis of ECM protein production, the cell growth media was supplemented with ^{3}H-glycine

as described above. Three days following ^{3}H-glycine addition for peptide density studies and two days following ^{3}H-glycine addition for the remaining studies, cells were removed non-enzymatically using 25 mM ammonium hydroxide, then rinsed with 70% ethanol and dried. This process leaves intact the ECM elaborated by cells during culture. Sequential enzyme digestion was used to determine the gross composition of these matrices. Elaborated matrix was digested first with 200 U/ml trypsin, followed by 58 U/ml elastase (ICN, Costa Mesa, CA), and then 76 U/ml collagenase. The enzymes were dissolved in TEC buffer which consisted of 25 mM Tris HCl, 5 mM $CaCl_2$, pH 8.0 [14]. The trypsin and elastase incubation steps were for 4 h at 37°C, whereas the collagenase incubations were carried out overnight at 37°C. After the final enzymatic digestion (collagenase) any material remaining on the substrate was dissolved by incubation with 1 N NaOH for 4 h at 37°C. Aliquots were taken from each step of the digest for scintillation counting.

2.4. Cell adhesion on bioactive materials

Glass slides were prepared as described above. Cells were seeded onto the glass substrates as described above, except that 12-well FlexiPerm strips were used (0.635×0.79 cm). At 4, 8, 12 or 24 h, media was removed from three wells, the wells were rinsed with phosphate-buffered saline (PBS) and new media was added. The remaining attached cells were then counted by viewing under a phase contrast microscope (Nikon, model TMS-F, Melville, NY) fitted with an eyepiece grid at $200\times$ magnification.

2.5. Evaluation of exogenous factors to increase matrix protein production

Vitronectin was adsorbed to a 24-well tissue culture plate by incubating with a 5 μg/ml solution of vitronectin at room temperature overnight. The remaining vitronectin solution was then removed and the wells were rinsed with PBS. Cells were seeded onto the vitronectin-adsorbed and plain 24-well tissue culture plates as described above, except no ascorbic acid was added. Transforming growth factor beta (TGF-β) was added at 1 ng/ml to growth media of wells with and without adsorbed vitronectin. Analysis of cell numbers and ECM protein production was performed in triplicate for each condition as described above.

2.6. Statistical analysis

Data sets were compared using two-tailed, unpaired *t*-tests. *P*-Values less than 0.05 were considered to be significant.

3. Results

Glass surfaces were prepared with different surface concentrations of the peptide RGDS in order to evaluate the effect of peptide density on ECM production for cells grown on bioactive materials. In these experiments, RGDS surface concentrations of 0.5 and 2 nmol/cm^2 were utilized. The higher concentration was the maximum peptide density obtained by these surface-modification techniques. The amount of ECM produced by the cells was determined by performing a sequential enzyme digest as described above, measuring the incorporated ^{3}H-glycine. The amount of matrix produced on a per cell basis was then determined from the average matrix-derived cpm per well and the average number of cells per well. These results for HASMC, HUVEC and HDF are given in Fig. 1. It should be noted that significant differences in adhesion and proliferation rates were observed for each of the cell types evaluated, but similar trends for matrix production were observed. Although initial adhesion was greater for each of the cell types on the surfaces with 2 nmol/cm^2 RGDS, all cell types produced significantly less matrix on a per cell basis on the surfaces with higher peptide density.

Glass surfaces were also prepared with different peptides at the same surface concentration, 2 nmol/cm^2, in order to evaluate the effect of the different peptides with different adhesion strengths on ECM production. The peptides used were RGDS, YIGSR, VAPG, VGVAPG, and KQAGDV. RGES was used as a negative control. Aminophase glass was used as a non-specific control to account for the influence of serum-associated factors due

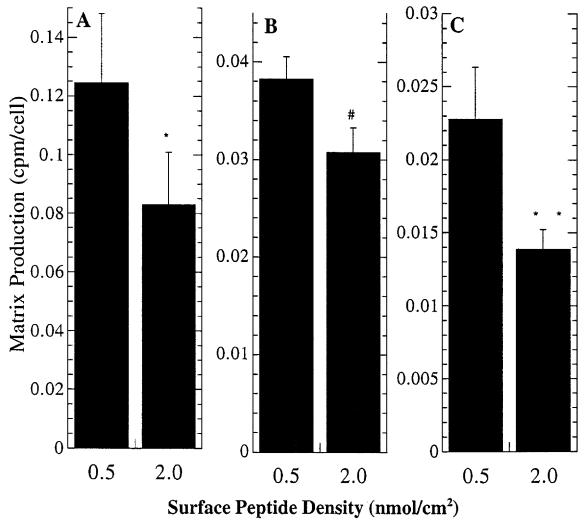

Fig. 1. Extracellular matrix protein production of cells grown on aminophase glass surfaces with covalently bound RGDS at varying surface concentrations. (A) HASMC, (B) HUVEC, (C) HDF. (*) $P = 0.03$ compared to 0.5 nmol/cm^2 RGDS surface. (#) $P = 0.005$ compared to 0.5 nmol/cm^2 RGDS surface. (**) $P = 0.003$ compared to 0.5 nmol/cm^2 RGDS surface. $N = 4$ per group.

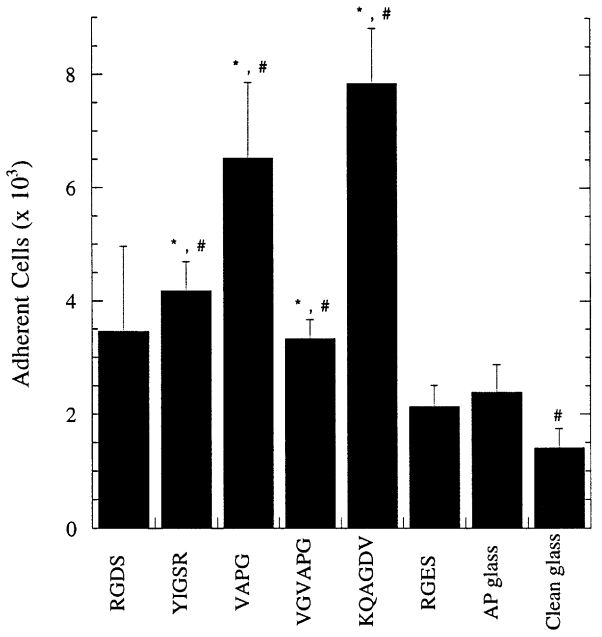

Fig. 2. Adhesion of SHR smooth muscle cells at 4 h to aminophase glass, clean glass, and aminophase glass with covalently bound cell adhesion peptides (2 nmol/cm^2). RGES was used as a negative control. (*) $P < 0.05$ compared to RGES. (#) $P < 0.05$ compared to AP glass. $N = 3$ per group. By 24 h, adhesion was similar for all surfaces except RGES and clean glass, where adhesion was significantly reduced.

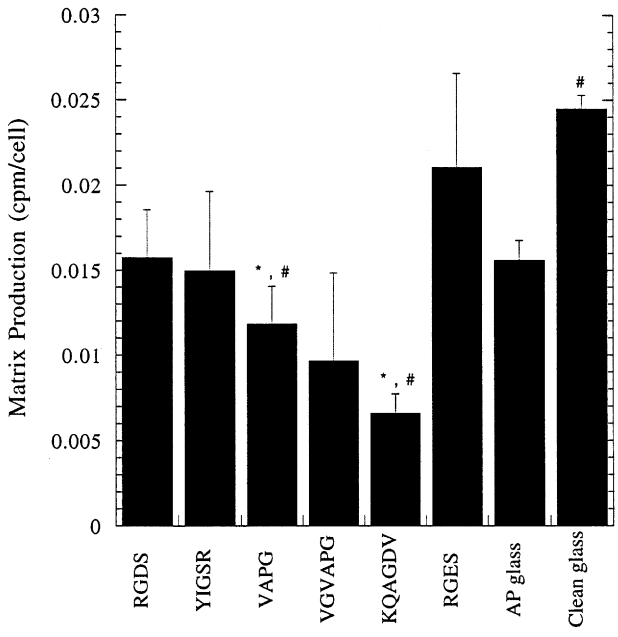

Fig. 3. Extracellular matrix protein production by SHR smooth muscle cells grown on aminophase glass, clean glass, and aminophase glass with covalently bound cell adhesion peptides (2 nmol/cm^2). RGES was used as a negative control. (*) $P < 0.05$ compared to RGES. (#) $P < 0.05$ compared to AP glass. $N = 4$ per group.

to the presence of FBS in the media. Adhesion was determined on the various surfaces at 4, 8, 12 and 24 h. The results of SHR adhesion to the various surfaces at 4 h are shown in Fig. 2. There was a greater amount of adhesion to all of the peptides (except RGES) than the control surfaces. At 8 and 12 h a transition began, such that by 24 h, there were no differences between the amount of adhesion on the non-specific control, aminophase glass, and the adhesive peptide-grafted surfaces. Similarities in later adhesion are likely due to the presence of serum proteins in the media. However, RGES and clean glass had significantly lower adhesion at 24 h than the remaining surfaces.

The results from analysis of matrix production on surfaces with different ligands are shown in Fig. 3. As with the peptide density study, the most adhesive surface, KQAGDV for SHR, displayed the least matrix production, while the least adhesive surface, RGES, had the greatest matrix production. This was also true for BAEC growing on the various peptides. Adhesion of BAEC on the various peptides was greater than on control surfaces at 4 h. Adhesion at 4 h was greatest on RGDS and YIGSR surfaces. By 24 h, however, adhesion to aminophase glass was similar to that seen with many of the peptides, while adhesion to RGES remained lower than the other surfaces. As seen in Fig. 4, matrix production on a per cell basis was again greatest on the least adhesive surface, RGES, and was lower on the more adhesive surfaces.

HASMC and BAEC were also grown in 24-well tissue culture plates with TGF-β, vitronectin, or both to determine if matrix production could be increased through the administration of exogenous factors. Matrix production on a per cell basis was determined as described above. These results are shown in Fig. 5. For HASMC, vitronectin and TGF-β each individually increased matrix protein production compared to that produced on tissue culture polystyrene (TCPS), and the combination of vitronectin and TGF-β further increased matrix production. For BAEC, TGF-β also increased matrix production over that produced on TCPS. However, vitronectin caused a decrease in matrix production, and matrix production with the combination of vitronectin and TGF-β was similar to that with TGF-β alone.

4. Discussion

The results using different surface peptide densities of RGDS showed that cells growing on surfaces with greater adhesivity produced less matrix. This was true for three different cell types used, HASMC, HUVEC, and HDF. The results using various peptides also indicated that a more highly adhesive surface results in decreased ECM production. These results were seen with two different cell types, rat aortic smooth muscle cells and bovine pulmonary artery endothelial cells. Although adhesion on the various surfaces was similar at 24 h,

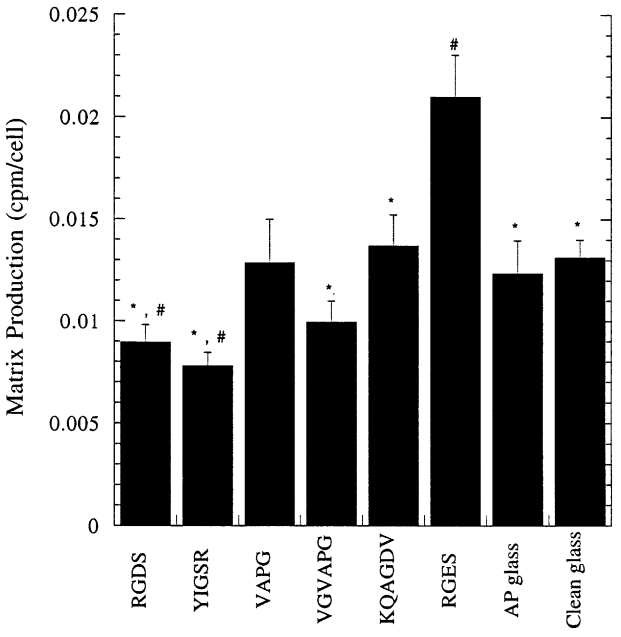

Fig. 4. Extracellular matrix protein production by BAEC grown on aminophase glass, clean glass, and aminophase glass with covalently bound cell adhesion peptides (2 nmol/cm^2). RGES was used as a negative control. (*) $P < 0.05$ compared to RGES. (#) $P < 0.05$ compared to AP glass. $N = 4$ per group. RGDS and YIGSR surfaces supported the greatest initial adhesion, while RGES surfaces displayed significantly lower adhesion even at 24 h.

differences in matrix production over two or three days were still observed. These results suggest that receptor–ligand interactions during adhesion may initiate signal transduction events to regulate ECM synthesis. Other factors may also influence matrix synthesis, such as stresses applied to the cytoskeleton [15]. In addition, the specific ligands used to produce bioactive materials could also have important implications as results have indicated that the matrix cells are grown on might control their phenotypic expression [16,17].

The amount of matrix produced by cells grown on biodegradable scaffolds is particularly important in tissue engineering applications, where scaffolds are expected to degrade over time. In order for the tissue to remain viable the scaffold must be replaced by ECM produced by the cells. If too little matrix is produced, the tissue may fail. Therefore, it may be important to optimize the amount of adhesion desired along with the amount of matrix production needed. However, there may also be ways to increase matrix production without decreasing initial adhesion to the scaffold. One method is through the addition of certain growth factors, which potentially could also be covalently bound to the scaffold [18]. Results shown here indicate that TGF-β is one growth factor that might help to increase matrix production in both smooth muscle cells and endothelial cells.

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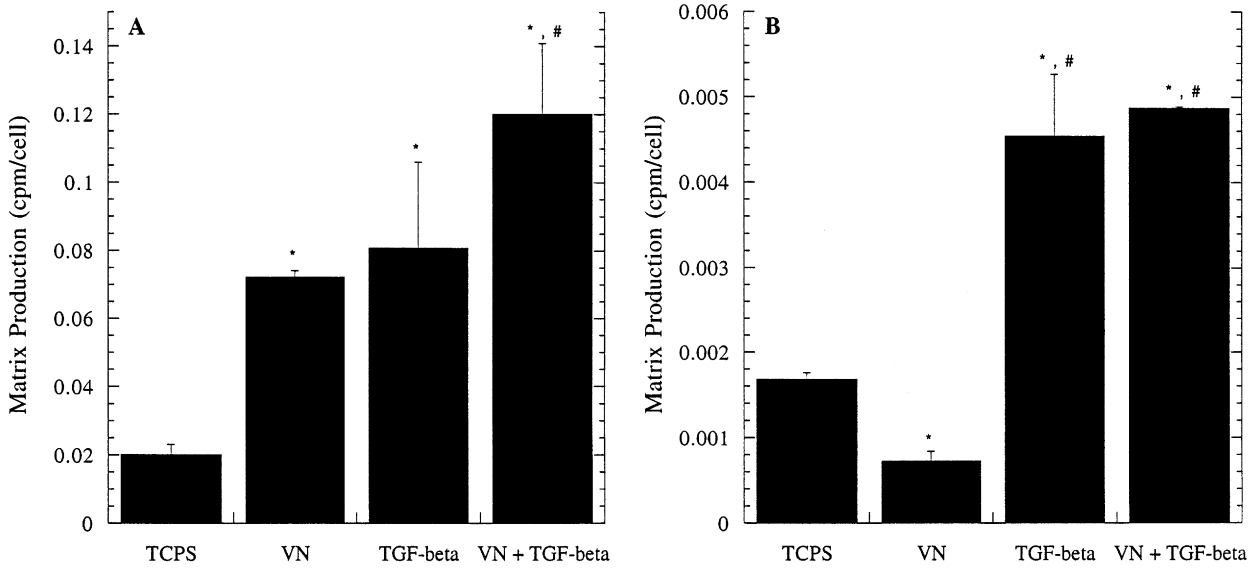

Fig. 5. Extracellular matrix protein production by cells grown on tissue culture polystyrene (TCPS) and TCPS with vitronectin adsorbed to the surface (VN), with 1 ng/ml transforming growth factor beta in the media (TGF-beta), or with both (VN + TGF-beta). (A) HASMC, (B) BAEC. (*) $P < 0.05$ compared to TCPS. (#) $P < 0.05$ compared to VN. $N = 3$ per group.

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