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RGD and YIGSR synthetic peptides facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes

AQ: 1

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Submitted 26 April 2004; accepted in final form 9 September 2004

Boateng, Samuel Y., Syed S. Lateef, William Mosley, Thomas J. Hartman, Luke Hanley, and Brenda Russell. RGD and YIGSR synthetic peptides facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes. *Am J Physiol Cell Physiol* 288: C000–C000, 2005. First published September 15, 2004; doi:10.1152/ajpcell.00199.2004.—In the mammalian heart, the extracellular matrix plays an important role in regulating cell behavior and adaptation to mechanical stress. In cell culture, a significant number of cells detach in response to mechanical stimulation, limiting the scope of such studies. We describe a method to adhere the synthetic peptides RGD (fibronectin) and YIGSR (laminin) onto silicone for culturing primary cardiac cells and studying responses to mechanical stimulation. We first examined cardiac cells on stationary surfaces and observed the same degree of cellular adhesion to the synthetic peptides as their respective native proteins. However, the number of striated myocytes on the peptide surfaces was significantly reduced. Focal adhesion kinase (FAK) protein was reduced by 50% in cardiac cells cultured on YIGSR peptide compared with laminin, even though β_1 -integrin was unchanged. Connexin43 phosphorylation increased in cells adhered to RGD and YIGSR peptides. We then subjected the cardiac cells to cyclic strain at 20% maximum strain (1 Hz) for 48 h. After this period, cell attachment on laminin was reduced to ~50% compared with the unstretched condition. However, in cells cultured on the synthetic peptides, there was no significant difference in cell adherence after stretch. On YIGSR peptide, myosin protein was decreased by 50% after mechanical stimulation. However, total myosin was unchanged in cells stretched on laminin. These results suggest that RGD and YIGSR peptides promote the same degree of cellular adhesion as their native proteins; however, they are unable to promote the signaling required for normal FAK expression and complete sarcomere formation in cardiac myocytes.

AQ: 2 cell adhesion; connexin43; focal adhesion kinase

THE EXTRACELLULAR MATRIX (ECM) plays an important role in regulating cell adhesion, migration, and proliferation in tissues as well as bearing mechanical stress. The interaction between cells and the ECM is important in embryonic development, growth, and disease. In the myocardium, fibroblasts make up about two-thirds of the total cell number (18). These fibroblasts are known to synthesize many of the ECM proteins, including fibronectin, vitronectin, and collagens type I, II, and V. Fibroblasts also produce much of the matrix metalloproteinases required for ECM degradation and remodeling during growth and development (11).

There are many ECM proteins, including the collagens, fibronectin, laminin, and elastin, to name but a few. Fibronectin molecules consist of three homologies that, in combination, have different cellular and ECM protein binding capabilities (15). These homologies, in addition to collagen and glycosaminoglycans, bind to cells through the RGD amino acid sequence. The laminins, on the other hand, are important components of the basement membrane and are composed of α -, β -, and γ -chains. Laminin can bind collagen IV, erican, and entactin and is thought to play a role in initiating the synthesis of the basement membrane (19). It also contains an RGD sequence on the α -chain and YIGSR on the β -chain, which facilitate cellular attachment. Although the ligands for cellular attachment are only a few peptides long, the complete size of these various ECM proteins is usually several thousand peptides in length. The exact role of the remaining regions of these large molecules is largely unknown.

The ECM is often seen as a component of the tissue synthesized and regulated by the cells within it, but many cellular activities can also be regulated by the ECM by a process known as “outside-in signaling” through the cell surface integrins (26). For example, in endothelial cells, synthesis of endothelin-1 can be influenced by the type of collagen the cells are cultured on (9). In the myocardium, the ECM plays an important role in ventricular remodeling following mechanical overload and hypertrophy (20).

In vitro, neonatal myocyte cultures are frequently used to study the influence of mechanical stimulation on cell signaling and myocyte remodeling. However, a significant number of cells become detached in response to mechanical stimulation when ECM proteins are noncovalently adsorbed onto the culture surface, limiting the scope of such studies. It would be advantageous to firmly adhere the ECM to the culturing surfaces so as to better withstand the effects of mechanical stimulation.

In this study, we have successfully adhered RGD and YIGSR synthetic peptides to the surface of silicone membranes for the purpose of cell culture. Cells cultured on these peptides were compared with their respective native peptides, fibronectin and laminin. This was done to determine the importance of the binding ligands on myocyte cellular adhesion, cell morphology, and function in the absence of the large non-cellular binding domains of these proteins. We sought to determine whether covalently adhered peptides could better withstand

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mechanical stimulation compared with their noncovalently adsorbed native proteins. We also determined the effect of these synthetic peptides on cell behavior and whether they could evoke functional downstream signaling in response to mechanical stimulation.

MATERIALS AND METHODS

Silicone membranes and modification of surface chemistry. Silicone membranes for cell culture were prepared, and surface chemical modification was performed as described previously (17, 23). The chemical steps used to adhere peptides to silicone are shown in Fig. 1A. Briefly, flat silicone membranes were made by mixing the base elastomer component and the cross-linker curing agent (Dow Corning) in a 10:1 ratio. The mixture was placed in either tissue culture polystyrene dishes (TCP; Falcon) or Flexcell six-well silicone culture

dishes and allowed to cure as suggested by the manufacturer. The membranes were then acid treated with 12 N HCl and coated with fibronectin or laminin as described previously (23). For surface chemistry, silicone membranes were either sonicated or shaken for 1 h in 12 N HCl. After the incubation step, the surface was washed and incubated for multiple cycles with deionized water and then placed in an oven at 60°C for 1–2 h. The amine surface was prepared as described previously (17), using 5% (3-aminopropyl)triethoxysilane (APTES) in 95% ethanol. The amine surface was stored under nitrogen and used within 1 day. The surface was subsequently treated with 0.2 mM sulfo-SMCC (Pierce) cross-linker, followed by different peptide concentrations up to 100 μM peptide incubation for 20 h at 4°C before culture.

The sequence of the peptides adhered to the silicone surfaces was CGGEGYGEGRGDSPG, the integrin-binding sequence found most commonly in fibronectin, and CGGEGYGEGYIGSR, for laminin. The thiol group on cysteine (C) was used to attach the peptide to the silicone. The residues between C and either RGD or YIGSR were added to extend the peptide off the surface to allow access to the cell surface integrins as well as to enhance the overall solubility of the peptide. The COOH terminus was aminated and the NH₂ terminus acylated to reduce peptide cleavage by cells during culture. The membranes were sterilized in 70% ethanol for 1 h, rinsed with Moscona’s buffer, and plated with myocytes. For the preparation of adsorbed protein surfaces, laminin or fibronectin protein was adsorbed on the silicone surface after acid treatment at a concentration of 25 μg/ml in DMEM buffer as described previously (23).

Myocyte and fibroblast cell cultures. Primary heart cultures were obtained from neonatal rats according to Institutional Animal Care and Use Committee and National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals” (NIH publication #85–23 Rev. 1985). Hearts were removed from 1- to 2-day-old neonatal Sprague-Dawley rats and placed in Moscona’s saline (136.8 mM NaCl, 28.6 mM KCl, 11.9 mM NaHCO₃, 9.4 mM glucose, and 0.08 mM NaH₂PO₄, pH 7.4) on ice. The atria were removed, and the ventricles were washed several times with cold Moscona’s buffer and then with cold Krebs Ringer buffer (KRB: 118.4 mM NaCl, 2.4 mM MgSO₄, 4.7 mM KCl, 23.8 mM NaHCO₃, 1.5 mM KH₂PO₄, and 11.1 mM glucose) with 1 mg/ml BSA fraction V and stock antibiotic/antimycotic solution diluted 1:100 (containing penicillin G/streptomycin/amphotericin; Sigma). Phenol red was used as a pH indicator, and the solution was gassed with 5% CO₂, pH 7.4. The tissue was minced with dissecting scissors in this modified KRB solution.

The cells were dissociated at 37°C in a shaking water bath for 10-min periods at 50 oscillations/min with a collagenase type 2 at 0.42 mg/ml (Worthington Biochemical) in the modified KRB solution, except that we used a higher concentration of BSA fraction V (20 mg/ml). During the digestion, triturating the tissue through a cannula/syringe mechanically disrupted the tissue. The cells released after the first digestion were discarded (fibroblast- and debris-rich mixture), whereas the cells from subsequent digestions were added to 25 ml of KRB with the higher BSA concentration and kept on ice. After final collection, the cells were pelleted by centrifugation (5,000 rpm for 6 min at room temperature) and the supernatant was discarded. The cells were resuspended, filtered through a metal sieve to remove large material, and preplated in different media as described below.

For fibroblast-enriched cultures, we compared two media. One group of cells was resuspended, plated, and maintained in PC1 medium (Biowhittaker/Cambrex) containing L-glutamine, antibiotic/antimycotic solution (Sigma), and gentamicin (50 mg/l). The other group was placed in DMEM, which contained Nutrient Mixture F-12 Ham without L-glutamine (Sigma), standard amino acid concentrations plus palmitic (2.56 mg/l) and linoleic (0.84 mg/l) fatty acids, antibiotic/antimycotic solution, gentamicin (50 mg/l), and 5% serum. The resulting cell mixtures were preplated in either media for 1 h in a 37°C CO₂ incubator. The adhered fibroblasts were cultured for another 2 days before passaging either PC1 serum-free medium or

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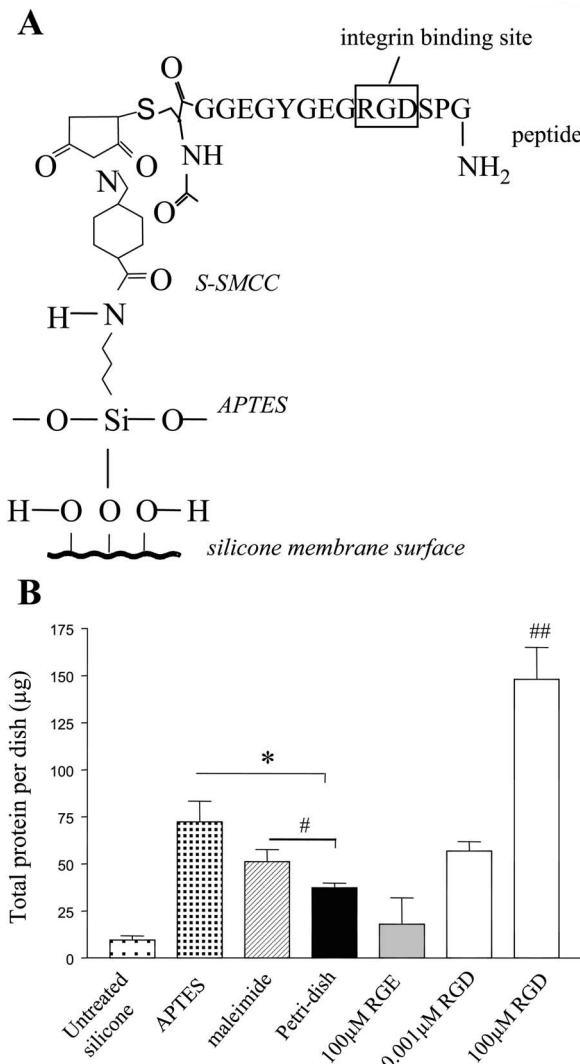


Fig. 1. A: diagram of a silicone surface with the various chemical groups needed to covalently link peptides to the surface. S-SMCC, sulfo-SMCC; APTES, (3-aminopropyl)triethoxysilane. B: total protein per culture dish from cardiac myocytes cultured on synthetic peptides adhered to silicone. With the RGD peptide, there was a significant increase in cell attachment in response to increased peptide concentration (##P < 0.01, 0.001 μM RGD vs. 100 μM RGD). The control peptide RGE did not facilitate much cellular attachment. The intermediate reaction steps APTES and maleimide also had higher attachment compared with the polystyrene culture dish. *P < 0.01, APTES vs. polystyrene; #P < 0.05, maleimide vs. polystyrene (n = 3 cultures).

DMEM complete medium with 5% serum. Fibroblast cell number was assessed by cytometric counting after 1 and 5 days following trypsinization. The counts from four dishes were averaged to assess cell number on each surface.

For myocyte-enriched cultures, we used the PC1 medium for resuspension and preplating. The cells remaining in suspension were plated at high density (1,000 cells/mm²) and kept for 1 day in PC1 medium (with the additions as noted above), after which time only a small percentage of fibroblasts still remained yielding a myocyte-enriched culture. PC1 prevented further proliferation of any remaining fibroblasts during this time. After 1 day in PC1, the myocyte-enriched cells were kept in DMEM-M199 (4:1) for the remainder of their time in culture.

Mechanical cyclic stain of cultured cells. Myocytes were mechanically stretched after 48 h of culture with the use of the Flexcell strain unit (model FX-4000; Flexcell International) as described previously (17). Cells were cyclically strained at 20% maximum strain at 1 Hz for 48 h in PC1 serum-free medium to limit nonmyocytic proliferation. After this period, cells were rinsed with Moscona's buffer before undergoing further analysis.

Protein analysis by Western blotting. Protein preparations, Western blots, and their analysis were performed as described previously (4). We measured the expression of β_1 -integrin (Research Diagnostics), connexin43 (Transduction Laboratories), focal adhesion kinase (FAK; Upstate Biotechnology), type 1 collagen antibody [Developmental Studies Hybridoma Bank (DSHB)], and myosin heavy chain (DSHB) in primary neonatal myocyte cultures. Peroxidase-conjugated secondary antibodies of donkey anti-goat, donkey anti-mouse, and donkey anti-rabbit (Research Diagnostics) were used. Proteins were then visualized using the ECL chemiluminescence system (Amersham).

Immunocytochemical staining. Immunostaining was performed as previously described (3) using anti-FAK antibody (Upstate Biotechnology), phalloidin (Molecular Probes), anti-connexin43 (Transduction Laboratories), anti-myosin (Iowa Hybridoma Bank), and appropriate fluorescence-labeled secondary antibodies. Counterstaining for actin was done using either Alexa-488-phalloidin (Molecular Probes) or rhodamine-phalloidin (Molecular Probes). The rhodamine-conjugated phalloidin was kept relatively low (1 in 1,000) so that nonstriated actin staining in nonmyocytes was minimal. Membranes were then mounted on glass slides with the addition of Vectashield with 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories) as a nuclear stain and an antifade reagent. Stains were visualized using a fluorescence microscope (Nikon Microphot-FXA), and images were digitally captured with a Spot RT color camera (Diagnostic Instruments).

Statistical analysis. Data are presented as means \pm SE. Sample number (*N*) was defined as the number of separate cultures performed at a different time from different animals. Data groups were compared using one-way ANOVA followed by a Student-Newman-Keuls multiple comparison test. Significance was taken at $P < 0.05$.

RESULTS

Assessment of cellular adhesion to synthetic peptides on silicone. After the attachment of peptides to silicone, cardiac neonatal myocytes were cultured on these dishes for 48 h before analysis. After this period of culture, total protein was measured from each dish as a measure of cell number to determine the concentration of reaction peptide that produced the highest cellular attachment. The peptide reaction concentration represents the concentration of peptide incubated on the treated silicone surface. The reaction concentration has been shown to alter the final surface concentration of the peptide (30). Cells were not trypsinized and counted because myocytes are very sensitive to trypsin, and viable cell number would have been significantly reduced as a result.

Figure 1B shows that untreated silicone is very hydrophobic and had the lowest cellular attachment as measured by total protein per dish. Cells plated on the intermediate reaction steps of APTES and maleimide showed significantly higher levels of total protein compared with a normal commercial polystyrene tissue petri dish. These results were then compared with synthetic RGD peptide at two reaction concentrations. The results show that increasing the RGD peptide reaction concentration significantly increases the degree of cellular attachment. To determine whether the cellular attachment was due to the RGD sequence, we also plated cells on 100 μ M RGE as a negative control in which aspartic acid had been replaced by glutamic acid. With the RGE peptide, the cellular attachment was very low and similar to that of untreated silicone. This also shows that the increased protein measurement was derived from the attached cells and was not due to indirectly measuring the attached peptide from the silicone surface.

Cells were subsequently plated on RGD and YIGSR peptides at varying concentrations and compared with the native proteins fibronectin and laminin, respectively. These results are shown in Fig. 2A. The density of cells on fibronectin was significantly higher than on laminin, as reflected in the total protein per dish. There was a steady increase in the cellular attachment as peptide concentration was increased for both RGD and YIGSR. At 100 μ M, cellular attachment was identical to that of their respective native proteins. At this concentration, cellular attachment on the RGD peptide was also significantly higher than on YIGSR.

Although cellular attachment was similar between peptide and their native proteins, there were significant morphological differences, as shown in Fig. 2, B–E. Figure 2, B and D, show neonatal cardiac myocytes cultured on laminin and fibronectin, respectively, stained for actin with rhodamine-phalloidin. Nuclei were stained with DAPI. The striated myofilaments can be seen clearly in myocytes cultured on these two surfaces. Blue nuclei without surrounding actin staining represent fibroblasts that contain significantly lower levels of actin compared with myocytes. Figure 2, C and E, show myocytes on YIGSR and RGD peptides, respectively. In both of these peptides, cells showed significantly fewer muscle striations after 2 days of culture and did not look very myocytic morphologically.

To assess the contribution of nonmyocytes in the culture to our experimental findings, we plated some cardiac fibroblasts in serum and PC1 serum-free medium. Figure 3A shows that after 5 days in culture, cardiac fibroblasts did not proliferate in PC1 medium. However, in serum medium, there was a fourfold increase in cell number from 1 day to 5 days of culture. To assess the possible contribution of nonmyocytic ECM production to myocyte cultures, we measured procollagen I by Western blotting, as shown in Fig. 3B. In cardiac cells cultured in PC1 medium, there was no detectable collagen I, unlike cells cultured in serum medium, which produced significant amounts of the protein. Subsequently, myocytes were cultured in PC1 medium to reduce the effects of fibroblast contamination. Therefore, in myocytes cultured in PC1, cell number after 2 days was a reflection of cell adhesion and not cell proliferation.

Focal adhesion proteins and ECM proteins. Myocytes on laminin and fibronectin were identical, so subsequent experiments were done by comparing laminin and its YIGSR peptide. Myocytes on laminin and YIGSR peptide were stained for the

F4 integrin protein FAK, as shown in Fig. 4, A and B. There was significantly more staining for FAK in myocytes cultured on laminin than on YIGSR peptide. Subsequent Western blotting also showed that FAK protein was reduced by ~50% in

myocyte cultures on YIGSR compared with laminin, as shown in Fig. 4E. Western blotting was also used to determine protein distribution and levels of the transmembrane protein β_1 -integrin. In this case, the protein became less punctate (Fig. 4D),

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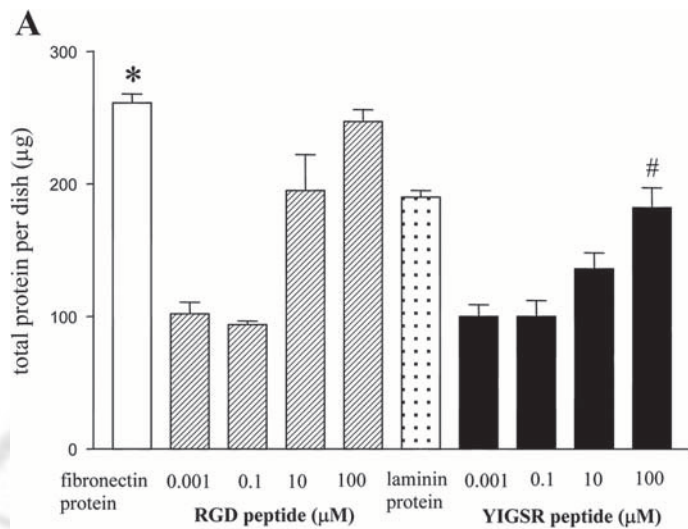
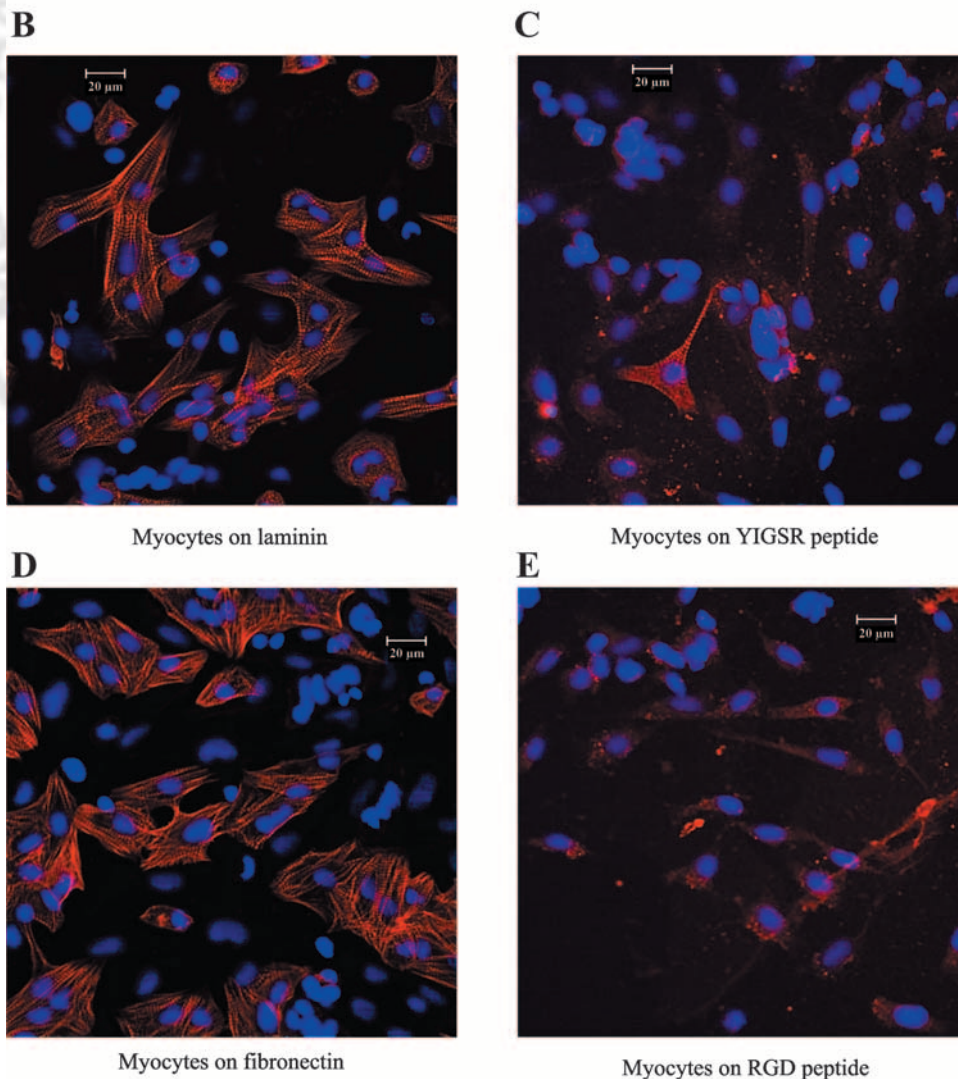


Fig. 2. A: myocyte attachment on synthetic peptides and their respective native proteins. Increasing concentrations of both RGD and YIGSR peptide increased cellular attachment. At the highest peptide concentration, attachment was comparable to that on fibronectin and laminin, respectively. Values for fibronectin and RGD were higher than for laminin and YIGSR. # $P < 0.05$, YIGSR vs. RGD; * $P < 0.01$, laminin vs. fibronectin ($n = 3$). B–E: immunofluorescence of neonatal rat myocytes cultured on laminin (B), YIGSR peptide (C), fibronectin (D), and RGD peptide (E). Cells were stained with phalloidin (red) for actin and with 4',6'-diamidino-2-phenylindole (DAPI) for nuclei (blue). Myocytes cultured on fibronectin and laminin had considerably more muscle striations compared with those cultured on RGD and YIGSR peptides. Calibration bar, 20 μ m.



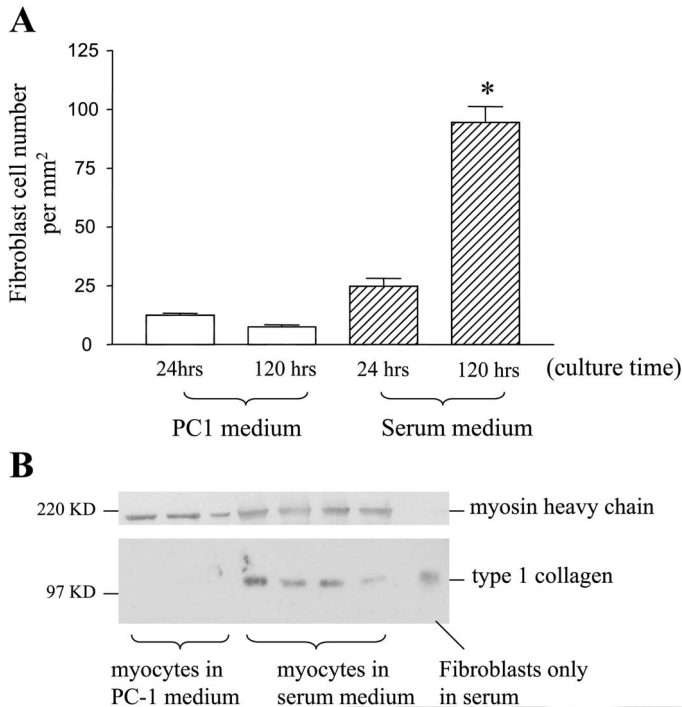


Fig. 3. Contribution of nonmyocytes to cardiac cultures. *A*: fibroblasts cultured in PC1 medium did not proliferate compared with those grown in serum-containing medium. * $P < 0.01$, serum at 24 h vs. serum at 120 h ($n = 4$ cultures). *B*: Western blot of myosin and type 1 collagen in cardiac cultures grown in either PC1 or serum medium. This fibroblast protein is absent from cells cultured in PC1 medium but is clearly present in those from serum medium.

but there was no significant difference in levels between myocytes cultures on laminin and YIGSR, as shown in Fig. 4*F*.

ECM and connexin43 phosphorylation in cardiac myocytes. Connexin43 phosphorylation was assessed using Western blotting and is shown in Fig. 5*A*. Connexin43 is represented by three main bands that migrate at different speeds in polyacrylamide gels. The highest band represents the most phosphorylated, whereas the lowest band represents the least phosphorylated. Cardiac myocytes cultured on both RGD and YIGSR peptides showed predominantly the upper two most phosphorylated bands, with reduced levels of the lower third band. In cells cultured on laminin, however, there was a more even distribution of the three bands, and subsequently, there was more of the dephosphorylated form. In the adult heart ventricle, this lower, least phosphorylated band (shown in the last lane of Fig. 5*A*) was also the predominant form, and the two upper, more phosphorylated forms were absent. In Fig. 5*B*, neonatal cardiac myocytes cultured on laminin are compared with connexin from adult rat atrium. In this case, the two upper, more phosphorylated bands appeared, whereas the lowest, least phosphorylated band was absent. Thus myocytes on synthetic peptides alter their connexin43 phosphorylation to a more adult atrial phenotype, whereas the native laminin protein produces both the atrial and ventricular forms.

Mechanical cyclic stretch of neonatal myocytes. Myocytes were cultured on synthetic peptides and laminin in Flexcell six-well dishes and cyclically stretched for 48 h at 1 Hz, 20% maximum strain. This was performed to determine whether covalently linking peptides to silicone improved cellular at-

tachment in response to mechanical stimulation. Figure 6*A* shows that there was almost a 50% decrease in total protein per dish after 48 h of cyclic stretching on laminin protein. In the chemically adhered group of RGD and YIGSR, cyclic stretching did not significantly alter the total protein per dish. Therefore, covalent attachment of peptides prevented the cellular detachment associated with mechanical stimulation, compared with those stretched on silicone coated with laminin. To determine whether the detachment of cells was due to detachment of the underlying matrix, we stretched myocytes on coated laminin dishes for 48 h, as shown in Fig. 6*B*. There was no difference in the amount of attached laminin on silicone after cyclic stretch. These results suggest that the detachment of cells from this surface after cyclic stretch was unlikely to be due to detachment of the underlying laminin.

To determine whether the cellular response to mechanical stimulation was similar between whole protein and synthetic peptides, we measured total myosin heavy chain from the cultured myocytes using Western blotting. Figure 6*C* shows that in neonatal myocytes cultured on laminin, there was no significant alteration in total myosin heavy chain when expressed as the ratio of total protein following mechanical stimulation. However, in cells cultured on YIGSR, there was a significant reduction in the expression of myosin after 48 h of mechanical stimulation. These data show that laminin and YIGSR promote different cellular responses to mechanical stimulation.

DISCUSSION

In this study, we have shown that cellular attachment to the ECM can be decoupled from the normal signaling events that frequently result from adhesion. We have shown that covalently bound RGD and YIGSR peptides alone are sufficient to facilitate cellular attachment to the same degree as their respective native proteins. However, in the absence of other active domains of these proteins, the peptides are unable to induce normal levels of FAK expression, despite normal β_1 -integrin expression. This leads to abnormal sarcomere formation, as seen with reduced muscle striations and a blunted response to mechanical stimulation. This occurs despite the fact that the cells on the peptide surfaces adhere more strongly and better resist mechanical stimulation. These differences are likely due to the initial plating matrix and are unlikely to be due to any additional matrix production by contaminating nonmyocytes. This is because the serum-free medium conditions used significantly reduced both the proliferation and matrix production of fibroblasts.

The RGD peptide sequence is thought to be one of the most abundant cell integrin recognition sequences in adhesive ECM proteins (31). The size of these recognition sequences is small compared with many of the ECM proteins, but the exact role of the remaining regions and their contribution to cell homeostasis is largely unknown. In HeLa cells, cellular attachment to the RGD sequence requires the recruitment of proteins on the cytoplasmic domain of the integrin and an intact cytoskeleton (29). Our data show that myocyte attachment to the peptides was strong enough to resist 48 h of significant mechanical stimulation.

These data suggest that the strength of the integrin attachment is not compromised by the absence of the remaining

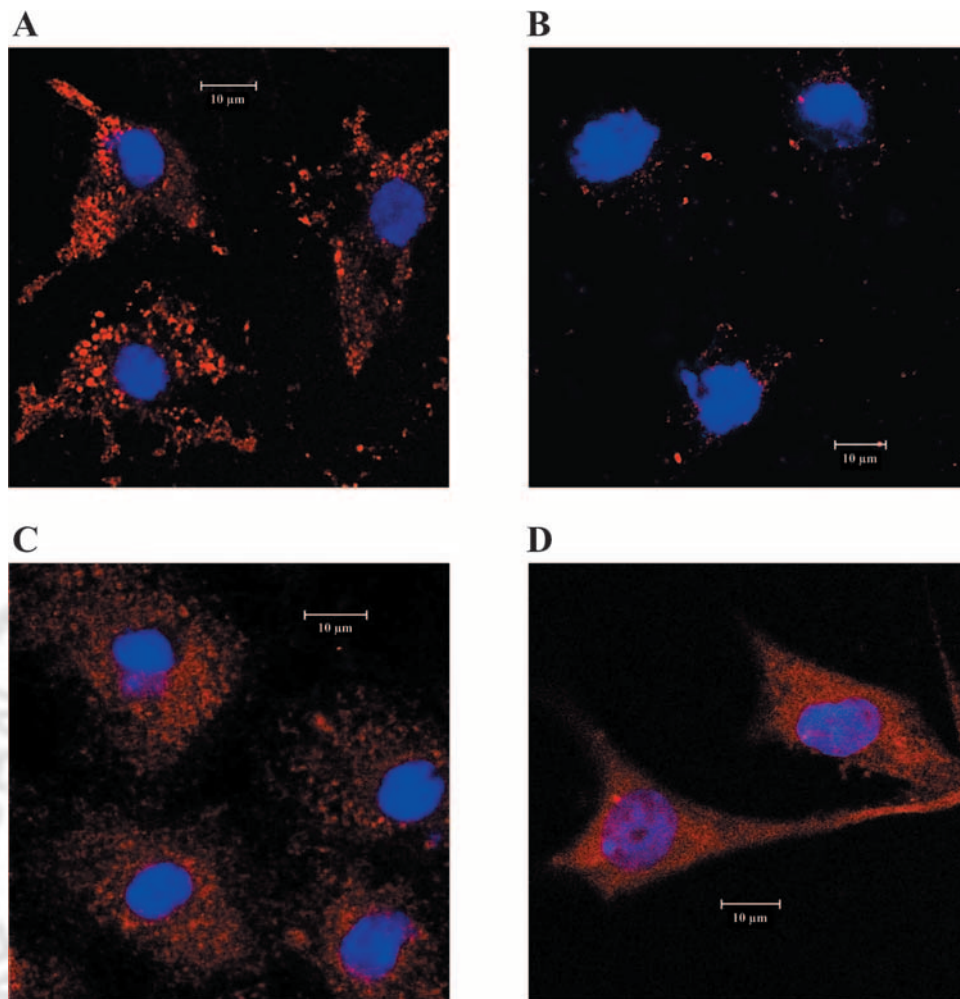
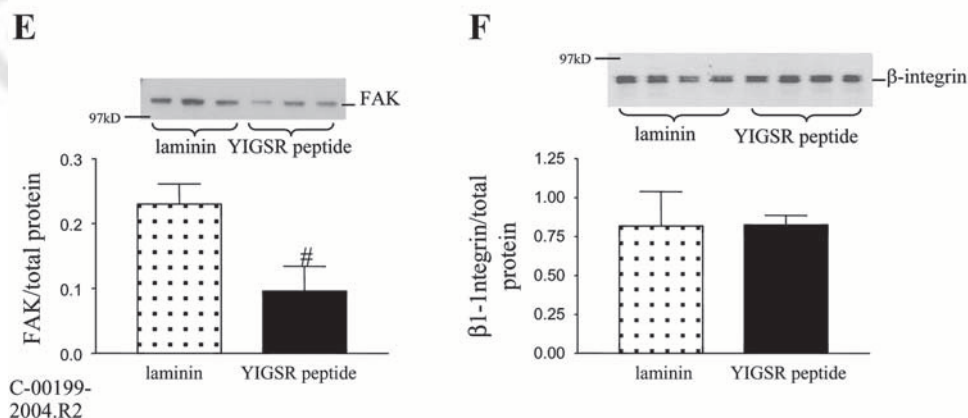


Fig. 4. Extracellular matrix (ECM) and focal adhesion proteins with examples of immunofluorescence staining in cardiac cells. *A* and *B*: focal adhesion kinase (FAK; red) and DAPI (blue) on laminin and YIGSR peptides. *C* and *D*: β_1 -integrin (red) on laminin and YIGSR peptides. Cells were cultured on laminin (*A* and *C*) or on YIGSR (*B* and *D*). FAK expression appears as punctate spots that are reduced in size and number for myocytes cultured on YIGSR peptide. β_1 -Integrin spots are also punctate on laminin but are more evenly distributed on YIGSR peptide. Calibration bar, 10 μ m. *E*: Western blot and quantitation of FAK amounts. The protein levels were reduced by $\sim 50\%$ in cells cultured on YIGSR peptide. $\#P < 0.05$, laminin vs. YIGSR ($n = 3$ cultures). *F*: Western blot and quantitation of β_1 -integrin. There was no difference between the protein levels in laminin and YIGSR ($n = 3$ cultures).



portions of the laminin or fibronectin proteins. The detachment of cells from laminin was not due to detachment of the underlying matrix, because cyclic stretch did not alter this. This finding suggests that the cellular attachment is different between laminin and its YIGSR peptide. This can be seen from the different distributions of β_1 -integrin in cells from the two groups. Investigators at our laboratory previously showed (17) that cardiac fibroblasts cultured on these synthetic peptides were also more resistant to trypsin compared with those on native proteins.

In 3T3-derived fibroblasts, the degree of receptor aggregation at the points of cellular attachment appears to be important for determining the strength of the adhesion to the RGD sequence (12). In one study, myocytes cultured on fibronectin were three times larger than those plated on noncoated dishes (26). This and other fibronectin-mediated effects could be inhibited in a dose-dependent manner with soluble GRGDSP. This would suggest that although the synthetic peptides can bind the cell surface integrins, they do not mediate the same signaling as the native proteins. It also has been suggested that

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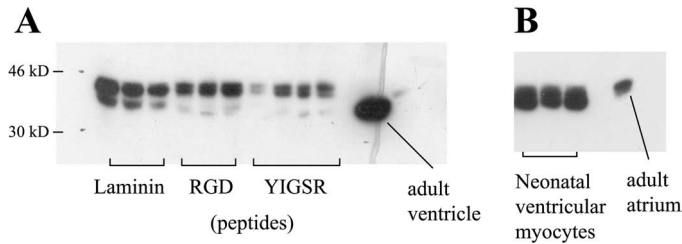


Fig. 5. ECM and connexin43 phosphorylation in myocytes. *A*: Western blot of connexin43 phosphorylation from myocytes cultured on laminin, RGD, and YIGSR. Three migrating variably phosphorylated forms of the protein appeared, with the most highly phosphorylated migrating the slowest. In cells cultured on laminin, all phosphorylated forms of the protein appeared in equal amounts. However, in cells cultured on both RGD and YIGSR, the least phosphorylated connexin43 was significantly reduced. This least phosphorylated form also appeared in the adult left ventricle. *B*: comparison between connexin43 in neonatal myocytes cultured on laminin and the protein from adult atrium. In the adult atrium, only the upper 2 more phosphorylated bands appeared, similar to what was found on the peptide cultures.

the unfolding of hidden coiled domains within ECM proteins during mechanical stimulation may play an important physiological role (8). Clearly, such complex dynamic changes within the matrix are not possible on the peptide surfaces. This may explain some of the differences in response to mechanical stimulation.

FAK localizes to sites of focal adhesions (33) and is important for maintaining cytoskeletal integrity, motility, and survival. Recruitment of FAK to the focal adhesions is part of the many signaling events that are thought to occur in response to integrin binding with the ECM. However, we show here that myocyte adhesion to YIGSR peptide does not induce the same degree of FAK expression as laminin. This results in formation of fewer striated myofibrils. Our data also show that the decreased FAK expression in myocytes cultured on YIGSR peptide is associated with an abnormal response to mechanical stimulation. FAK has been shown to be an important component in the early response to stretch in cardiac myocytes (28, 35). Work in our laboratory showed recently (21) that FAK is required for muscle sarcomere addition and myocyte remodeling in response to static stretch. Thus decreased FAK expression would be considered detrimental to myocyte homeostasis because it reduces the amount of phosphorylatable protein available to the cell. This in turn might affect potential signaling events required for an adequate response to mechanical stimulation.

In the myocardium, connexin43 plays an important role in cell-cell communication; however, the exact mechanism of this regulation is poorly understood (16). Our data show that connexin43 is more phosphorylated in myocytes cultured on synthetic peptides compared with the native proteins. Western blots also have been used to determine the phosphorylation of other phosphoproteins (2).

Atrial and ventricular myocytes show a different pattern of connexin expression in vitro (14). Our data show that there is also a different pattern of phosphorylation between the atrium and ventricle in the adult heart, with the atrium having more dephosphorylated connexin43. Physiologically, this is associated with higher gap junction communication; however, in vitro, the effects of connexin43 phosphorylation are less clear (1, 7, 16). In vivo, connexin43 can be phosphorylated by protein kinase A and protein kinase C through a number of

signaling pathways (1, 7, 16). As yet, the role of the ECM in connexin regulation in the heart is unknown. In the hypertrophied and failing heart, there are often significant changes within the ECM (10, 27), including changes to matrix protein isoforms (6, 36). This is associated with significant and frequently detrimental changes to connexin43 expression and function (13, 32, 34). Our data presented here show that

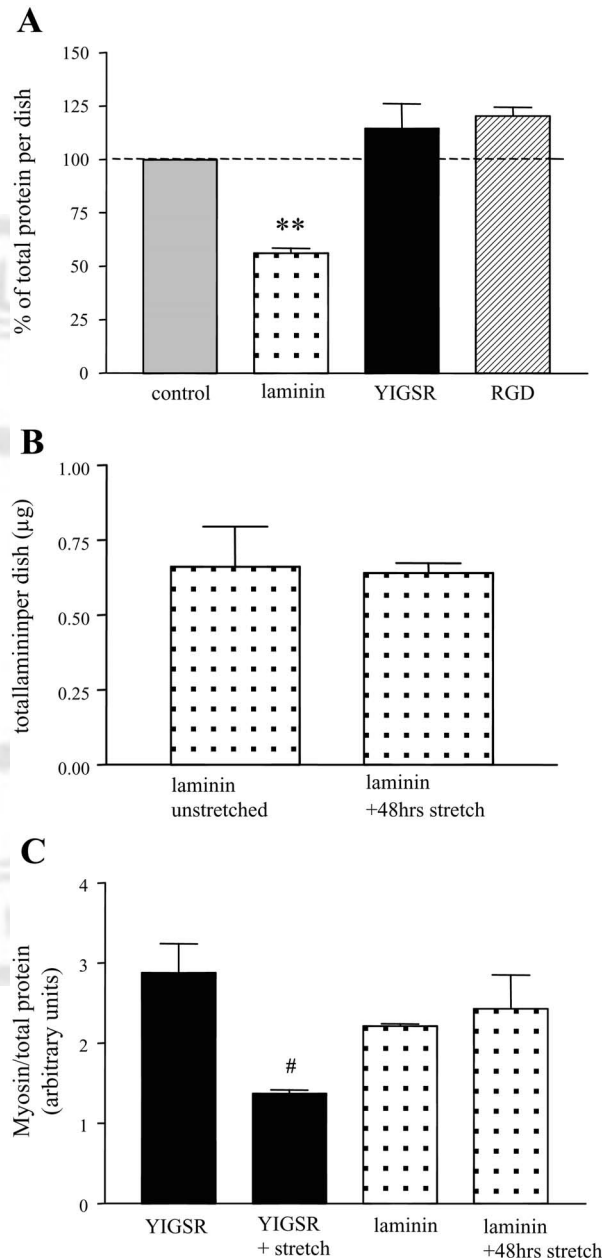


Fig. 6. Mechanical stimulation and covalently adhered synthetic peptides. *A*: percentage of cells remaining per culture dish after 20% cyclic stretch for 48 h at 1 Hz. After this period, there was no change in the number of cells attached to RGD and YIGSR peptides. However, on laminin, cell number was significantly reduced. ** $P < 0.01$, control laminin unstretched vs. laminin stretched ($n = 3$ cultures). *B*: amount of laminin adsorbed onto silicone surfaces with and without cyclic stretch. The amount of attached laminin was unchanged after 48 h of cyclic stretch. *C*: quantitation of Western blotting for myosin protein. Protein expression was unchanged after stretch in cells cultured on laminin but was significantly reduced in cells cultured on YIGSR peptide. # $P < 0.05$, control YIGSR unstretched vs. YIGSR stretched ($n = 3$ cultures).

synthetic peptides RGD and YIGSR increase the phosphorylation of connexin43 in myocytes, an event that would decrease gap junction communication. In alveolar epithelial cells, fibronectin has been shown to increase connexin43 expression and intercellular communication, compared with laminin-rich Matrigel (22). Numerous other studies also have shown that the ECM can directly and indirectly alter gap junctional communication in various cell types (5, 24, 25). These results, in combination with our own, strongly suggest that the ECM can influence both the expression and function of gap junctions. Our data present the first such findings in myocytes and may have some important implications in the failing heart, where significant changes to the ECM occur as part of the remodeling process.

In this study, we have been able to decouple the effects of adhesion to the ECM and subsequent downstream signaling required for FAK recruitment to the focal adhesion and cytoskeletal integrity. We show that RGD and YIGSR peptides are all that is required to achieve the same degree of myocyte adhesion as their respective native proteins, fibronectin and laminin. Moreover, these covalently adhered peptides provide better resistance to mechanical strain in cultured neonatal myocytes. However, in the absence of a large portion of the native protein, adhesion was unable to induce the same degree of FAK expression even though β_1 -integrin levels were unchanged. This was associated with abnormal sarcomere and myofilament formation in these cultured myocytes. Therefore, although these covalently adhered peptides provide improved cellular adhesion in response to mechanical stimulation, they promote aberrant myocytic gene expression and cell morphology.

ACKNOWLEDGMENTS

The MF20 myosin antibody (Fischman) was obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development, maintained by University of Iowa, Biological Sciences, Iowa City, IA 52242.

GRANTS

This research is supported by National Heart, Lung, and Blood Institute Grants HL-64956 and HL-62426 (to B. Russell).

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