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Control of Smooth Muscle Cell Proliferation and Phenotype by Integrin Signaling through Focal Adhesion Kinase

Alex O. Morla[1] and Jon E. Mogford[2]
Department of Pathology, Committee on Cancer Biology, University of Chicago, Chicago, Illinois 60637

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Extracellular matrix proteins such as fibronectin (FN) and laminin (LM) are known to help control the growth and phenotype of vascular smooth muscle cells (VSMCs). Here we have analyzed the relationship between growth factor and integrin signaling pathways in VSMCs. Culturing porcine coronary artery smooth muscle cells (PCASMCs) on FN and LM leads to distinct effects on cell proliferation and contractile protein expression. PCASMCs cultured on FN proliferate at a higher rate than cells cultured on LM, regardless of the growth factor used to support proliferation. Moreover, cells cultured on LM show higher levels of expression of smooth muscle myosin heavy chain (a marker of smooth muscle cell differentiation) than cells cultured on FN. In contrast to the effects on proliferation and contractile protein expression, both FN and LM supported cell migration in response to PDGF. Also, both FN and LM supported activation of ERK1 and ERK2 in response to PDGF and bFGF. However, FN and LM did show a difference in their ability to support signaling through the focal adhesion kinase (FAK). PCASMCs cultured on FN show robust activation of FAK in response to either PDGF or bFGF, however, cells cultured on LM show little-to-no activation of FAK in response to the growth factors. The results show that integrin signaling pathways have a profound effect on VSMC proliferation and phenotype, and that FAK is an important intermediate in these signaling pathways. The implications of our findings on the mechanisms controlling VSMC proliferation and phenotype in pathological states such as atherosclerosis and restenosis are discussed.

Key Words: **myosin; migration; ERK; MAPK; FAK; phosphorylation.**

Neointima formation resulting from vessel wall injury is a complex process that involves responses of vascular smooth muscle cells (VSMCs) and endothelial cells. In animal models the involvement of the VSMCs is often categorized into phases or waves (1, 2). The first wave is characterized by replication of the SMCs within the media followed by migration of the cells towards the intima (2nd wave) where the cells again proliferate to form the neointima (3rd wave). Each wave represents a complex cellular response by VSMCs to numerous external stimuli including loss of medial cell–cell integrity, altered extracellular matrix (ECM), growth factors, and plasma components (e.g., complement factors and plasma fibronectin) (3–5).

The VSMCs are normally surrounded by a basement membrane composed primarily of laminin, collagen IV, and heparan sulfate proteoglycan (6–8). The basement membrane provides structural continuity between the cells and vascular wall for transfer of contractile forces generated by the cells. The basement membrane also provides signals to the VSMCs maintaining the cells in the contractile phenotype—a response that in vitro has been duplicated to varying degrees using basement membrane components as adhesive substrates (i.e., matrigel, laminin) (9–15). After vessel wall injury, the integrity of the basement membrane is compromised as a result of the traumatic action or by increased proteolytic activity of responding VSMCs. The cells then encounter an ECM enriched in fibronectin (FN) from infiltrating plasma and later from cellular fibronectin expressed within the vascular wall.

Fibronectin is known to stimulate the migration and growth of numerous cell types including VSMCs (7, 8, 16, 17). These actions of fibronectin are mediated through cell surface receptors from the integrin family of proteins (18). Integrins are known to activate signaling pathways which include the activation of focal adhesion kinase (FAK), c-src, and the Ras/ERK pathway (19–22). Different ECM proteins interact with distinct integrins on cells. For example, SMCs adhere to FN

[1] To whom correspondence should be addressed at Department of Pathology, MC 1089, 5841 South Maryland Avenue, Chicago Illinois 60637. Fax: (773) 834-5251. E-mail: amorla@midway.uchicago.edu.
[2] Current address: Wound Healing Research Laboratory, Northwestern University, Chicago, IL 60611.

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primarily through the integrin $\alpha5\beta1$, while they adhere to LM through the integrins $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ (7). Thus, the adhesion of VSMCs to FN may lead to the activation of different signaling pathways than the adhesion of cells to LM. The signaling pathways activated by the adhesion of VSMCs to FN and LM have not been thoroughly investigated. In addition, the roles of these signaling pathways in the regulation of VSMC proliferation and phenotype are not well known. In this study we have investigated the effect of VSMC adhesion to FN and to LM on cell proliferation, cell migration, and on the activation of intracellular signaling pathways. We find that cell adhesion to FN supports cell proliferation and the activation of FAK, while cell adhesion to LM does not. These findings indicate that integrin signaling pathways have a profound effect on SMC proliferation and phenotype, and that FAK is an important intermediate in these signaling pathways.

MATERIALS AND METHODS

Materials. DMEM, glutamine Pen-Strep and mouse laminin were obtained from Life Technologies, Inc. FBS was purchased from HyClone Laboratories, Inc. Plasma fibronectin was purified from human plasma by gelatin-agarose affinity chromatography (23). The 4–20% gradient SDS–PAGE gels were from Novex. Anti-phospho-p44/42 MAPK polyclonal antibody was purchased from Promega. Anti-FAK mAb 2A7 and anti-pTyr mAb 4G10 were obtained from Upstate Biologicals, Inc. Protein A/G agarose was from Santa Cruz Biotechnology, Inc. ECL plus reagent and Hyperfilm were obtained from Amersham. Complete protease inhibitor cocktail tablets were purchased from Boehringer Mannheim Biochemicals. Boyden chamber 96-well apparatuses were obtained from Neuro Probe, Inc. Anti-smooth muscle myosin antibody (clone hSM-V) and all other chemicals were obtained from Sigma.

Cell culture. Primary porcine coronary artery smooth muscle cells (PCASMCs) were isolated from the coronary arteries of adult hogs essentially as described previously (24). PCASMCs were cultured in DMEM supplemented with 10% FBS and glutamine Pen-Strep. PCASMCs were typically between passages 2 and 7 for experiments.

Proliferation assays. Culture dishes were coated with 20 μg/ml FN or LM (in PBS) for 1 h at RT. Wells were washed 3 times with PBS, then blocked for 1 h at RT with 0.1% BSA in PBS before adding cells. PCASMCs were seeded onto the coated dishes in either complete medium (i.e., DMEM + 10% FCS) for the cell counting assay, or in DMEM + 0.5% BSA with either 2 ng/ml PDGF-BB or 5 ng/ml bFGF for the MTT assay. The cells were incubated at 37°C for 48 h, then the cells were either trypsinized and counted (Fig. 1A and Fig. 2), or MTT was added to the culture medium for 4 h and the amount of proliferation was measured as previously described (25, 26).

Immunoblotting assays. For the smooth muscle myosin assay cells from the counted proliferation assay (i.e., cells cultured in the presence of 10% serum, see above) were lysed in SDS–PAGE sample buffer and incubated at 100°C for 5 min. Samples were separated on 4–20% gradient Novex gels, transferred to Immobilon P membranes, blocked with 5% nonfat dry milk in TBS-Tween, then probed with anti-smooth muscle myosin antibody according to the manufacturer's recommendations. Blots were developed with the ECL plus reagent and exposed to Hyperfilm. Typical exposure times were under 5 min.

For the ERK activation assays, culture dishes were coated with 20 μg/ml FN or LM (in PBS) for 1 h at RT. Wells were washed 3 times with PBS then blocked with 0.5% BSA for 1 h at RT. PCASMCs were harvested by trypsinization and collected into DMEM + 0.5% BSA + soybean trypsin inhibitor. Cells were washed 3 times with DMEM + 0.5% BSA, then seeded onto the coated wells (10^5 cells per cm^2) and allowed to adhere for 2 h at 37°C. Cells were then stimulated with either 2 ng/ml PDGF-BB or 10 ng/ml bFGF for the length of time indicated in the figures. Cells were washed once with ice-cold PBS, lysed in 2× SDS–PAGE sample buffer and samples were incubated at 100°C for 5 min. Samples were separated on 4–20% gradient Novex gels, transferred to Immobilon P membranes, blocked with 5% nonfat dry milk in TBS-Tween, then probed with anti-phospho-p44/42 MAPK antibody according to the manufacturer's recommendations. Blots were developed with the ECL plus reagent and exposed to Hyperfilm. Typical exposure times were under 5 min.

Migration assay. Migration assays were done in a modified Boyden chamber assay essentially as described previously (27). Briefly, the membranes of 96-well Boyden chamber apparatuses were coated on both sides with either 20 μg/ml FN or 20 μg/ml LM in PBS, overnight at 4°C. PCASMCs (15,000 cells per well) in DMEM + 0.5% BSA were placed in the top reservoir, the bottom reservoirs contained DMEM + 0.5% BSA plus either no growth factor, or various concentrations of PDGF-BB or bFGF. Cells were placed at 37°C and allowed to migrate for 4 h, then the cells were fixed in 20% MeOH for 10 min at RT. Cells were stained with 0.5% toluidine blue, 20% MeOH for 10 min, RT followed by destaining in water. The cells on the top of the filter (i.e., the cells that had not migrated onto the bottom side) were scraped off with a cotton applicator, and the amount of cell migration was measured by placing the membrane apparatus in an ELISA reader and measuring the absorbance at 600 nm. We have determined that the Abs 600 is linearly proportional to the number of cells on the membrane in this type of assay.

FAK assay. Culture dishes were coated for 1 h at RT with either 20 μg/ml FN or 20 μg/ml LM in PBS, washed 3 times with PBS, then blocked with 0.5% BSA in PBS. PCASMCs in DMEM + 0.5% BSA were seeded onto the coated dishes and allowed to adhere for 2 h at 37°C. Cells were then treated with either no growth factor, or with 1 ng/ml PDGF-BB or 5 ng/ml bFGF for 10 min. Cell monolayers were washed once with ice-cold PBS then cells were lysed with IP Lysis buffer (1% NP40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM Na_3VO_4, 2 mM PMSF) (10^7 cells/ml) and lysates were clarified by centrifugation at 14,000*g* for 15 min at 4°C. Anti-FAK mAb 2A7 was added (5 μl 2A7 per ml of lysate; note that one sample received no anti-FAK antibody, as a negative control), samples were incubated at 4°C overnight, then 40 μl protein A/G agarose was added per sample and the samples were incubated with mixing for 3 h at 4°C. Samples were washed 4 times with IP Lysis buffer, then 40 μl of 5 × SDS–PAGE buffer was added and the samples were heated to 100°C for 5 min. Samples were separated on 4–20% gradient Novex gels, transferred to Immobilon P membranes, blocked with 1% BSA in TBS-Tween, then probed with anti-phosphotyrosine mAb 4G10 according to the manufacturer's recommendations. Blots were developed with the ECL plus reagent and exposed to Hyperfilm. Typical exposure times were under 5 min.

RESULTS

To test the effect of fibronectin (FN) and laminin (LM) on smooth muscle cell proliferation porcine coronary artery smooth muscle cells (PCASMCs) were seeded onto dishes coated with these matrix proteins and cell proliferation was measured. As shown in Fig. 1A, during the first 48 h after plating in the presence of serum, FN supported the proliferation of PCASMCs whereas LM only supported a small amount of cell proliferation. Similar results were obtained when cell

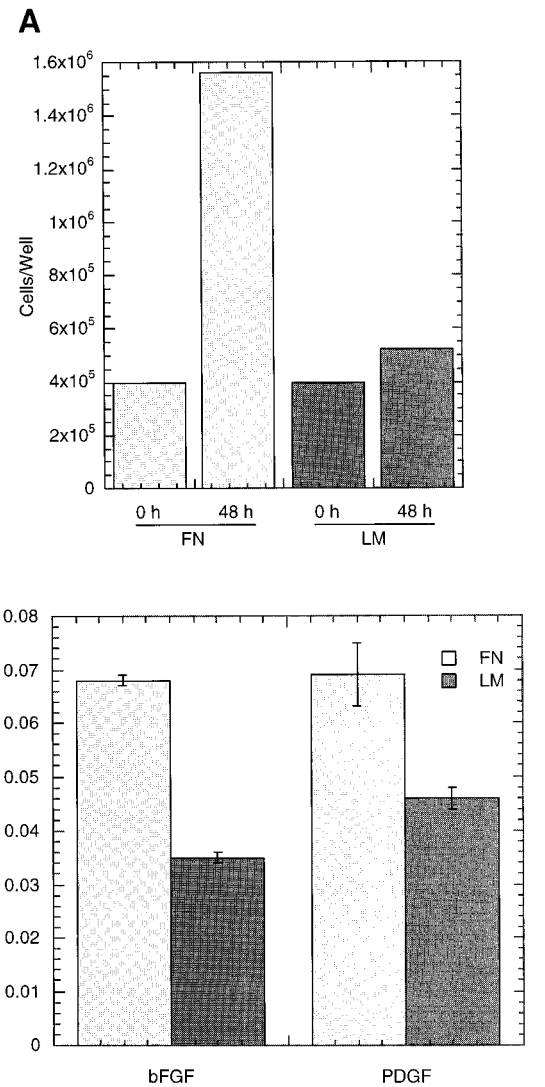

FIG. 1. Proliferation of PCASMCs cultured on FN and LM. (A) PCASMCs (4×10^5 per 10 cm dish) were cultured in complete medium on dishes coated with either 20 μg/ml FN (light gray bars) or 20 μg/ml LM (dark gray bars). After 48 h in culture the number of cells in each culture dish was counted. (B) PCASMCs in serum-free medium were cultured on either FN (light gray bars) or LM (dark gray bars) coated dishes in the presence of either 2 ng/ml PDGF-BB or 5 ng/ml bFGF. After 48 h the amount of cell growth was quantitated with the MTT assay. The bars represent the amount of cell growth at the 48 h time point. All samples were in triplicate. In both cases cells on FN grew better than cells cultured on LM.

proliferation was stimulated with the purified growth factors PDGF and bFGF (Fig. 1B).

Proliferation of smooth muscle cells is often accompanied by a change to a less contractile phenotype (1–5, 28). Consistent with this idea, culturing PCASMCs on FN resulted in lower expression of smooth muscle myosin heavy chain (a marker of smooth muscle cell differentiation) (Fig. 2). The results described above demonstrated that FN and LM exert different effects on

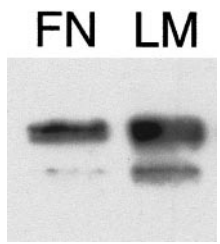

FIG. 2. Smooth muscle myosin expression in PCASMCs cultured on FN and LM. PCASMCs from the growth experiment (Fig. 1A) were analyzed by immunoblotting with anti-smooth muscle myosin heavy chain antibodies. Note that cells cultured on LM show higher levels of smooth muscle myosin protein than cells cultured on FN.

PCASMC proliferation and contractile protein expression.

Extracellular matrix proteins and growth factors are known to influence the migration of cells. The migration of PCASMCs was found to be strongly influenced by the growth factor used; cells migrated well in response to PDGF, but not in response to bFGF (Fig. 3). Interestingly, both FN and LM supported cell migration in response to PDGF. Therefore, although LM does not support rapid cell proliferation it does support cell migration in response to the appropriate growth factor.

Because FN and LM had different effects on cell proliferation we studied signaling pathways stimulated by

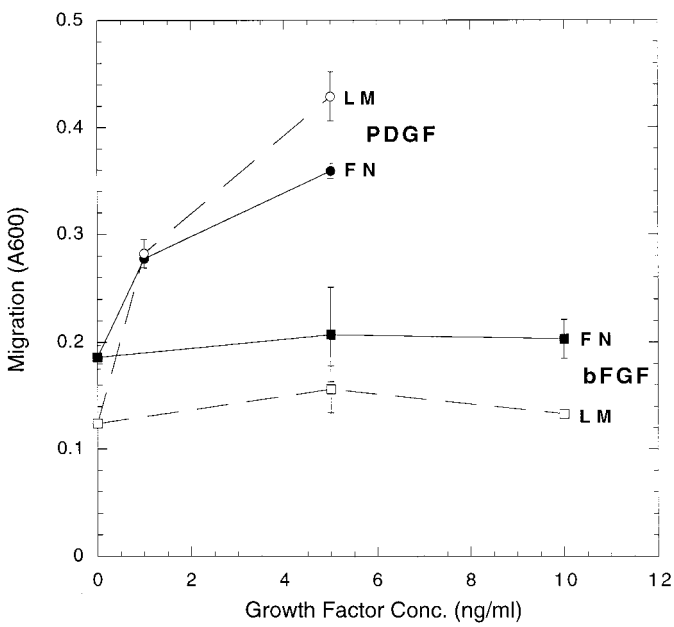

FIG. 3. PCASMC migration on FN and LM in response to PDGF and bFGF. PCASMCs in serum-free medium were placed in migration chambers that had membranes coated with either 20 μg/ml FN (●, ■) or 20 μg/ml LM (○, □). The lower chambers contained either PDGF-BB or bFGF at various concentrations to stimulate cell chemotaxis. Cells were allowed to migrate for 4 h, then the numbers of cells that migrated to the bottom side of the membrane were quantitated. All samples were in triplicate.

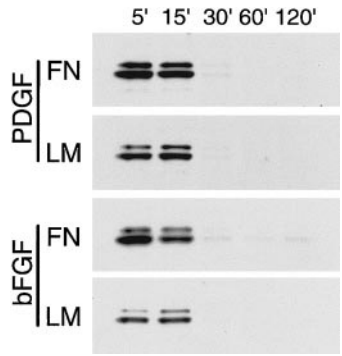

FIG. 4. Activation of ERK1/2 by growth factors in PCASMCs cultured on FN and LM. PCASMCs were cultured for 2 h on dishes that had been precoated with either 20 μg/ml FN or 20 μg/ml LM. PDGF-BB (2 ng/ml) or bFGF (10 ng/ml) were then added for either 5, 15, 30, 60, or 120 min (as indicated), cell lysates were collected and then analyzed by immunoblotting with anti-phospho-ERK antibodies.

these two matrix proteins. The first pathway analyzed was the Ras/MAP kinase pathway. PCASMCs cultured on either FN or LM were stimulated with either PDGF or bFGF and the activation of ERK1 and ERK2 was analyzed. As shown in Fig. 4, both PDGF and bFGF stimulated rapid and transient activation of ERK1/2 in PCASMCs. ERK1/2 phosphorylation was high at both 5 and 15 min after growth factor addition, and was reduced by 30 min after growth factor addition. These results suggest that signaling pathways activated by FN and LM are able to support growth factor induced ERK activation to similar levels.

The other signaling pathway analyzed was the Focal Adhesion Kinase (FAK) pathway, which has been shown in various systems to be controlled by integrin signaling (19). In contrast to the results obtained with ERK1/2 activation (shown above), FAK activity was differentially regulated by the matrix proteins. PCASMCs on FN showed robust FAK activation by both PDGF and bFGF whereas cells cultured on LM showed little or no activation of FAK in response to growth factors (Fig. 5). Note that cells cultured on FN in the absence of growth factors (the No GF lane) show no activation of FAK under these conditions. Thus FAK activation requires signaling by both the growth factor receptors and the FN-binding integrins. Thus, cell adhesion to FN, but not to LM, activates integrin signaling pathways that cooperate with growth factor signaling pathways, leading to FAK activation and cell proliferation.

DISCUSSION

In this study we have shown that culturing PCASMCs on FN and LM has distinct effects on cell proliferation and phenotype. Cells cultured on FN proliferate and express low levels of smooth muscle myosin, whereas cells cultured on LM proliferate at a lower rate and express high levels of smooth muscle myosin. In contrast to the effects on proliferation and myosin expression, both FN and LM supported cell migration in response to PDGF. In addition, both FN and LM supported growth factor-induced ERK1/2 activation. The one signaling difference that did parallel the proliferation results was that of FAK activation; FN adhesion supported growth factor-induced FAK activation, while LM did not. The results demonstrate that PCASMC proliferation and phenotype are strongly controlled by integrin signaling in a manner that parallels FAK activation.

Our data showing that FN and LM can differentially regulate PCASMC proliferation and contractile protein expression are consistent with previous work with SMCs (15). For example, Hedin *et al.* found that SMCs cultured on LM retained a contractile phenotype longer than cells cultured on FN (11, 12). In addition, Li *et al.* found differences in ERK1/2 activity in cells cultured on uncoated plastic as compared with cells cultured on Matrigel (a basement membrane protein preparation which contains LM and collagen IV, among other proteins) (29). Our results extend these previous findings by showing that one mechanism by which FN induces a proliferative, less contractile phenotype is by cooperating with growth factors to activate FAK. FAK activation may then lead to cell proliferation and modulation from a contractile phenotype to a synthetic phenotype.

Our data indicate that FN and LM are both able to support cell migration in response to PDGF stimulation. It is interesting to note that in animal models of neointima formation PDGF is thought to play an important role in stimulating SMC migration from the media to the intima, whereas bFGF is not thought to be important for migration (30–33). This is consistent with our findings that PDGF stimulates SMC migration whereas bFGF does not, irregardless of whether the cells are on FN or LM.

The connection between integrin signaling and ERK1/2 activation has been studied in many systems

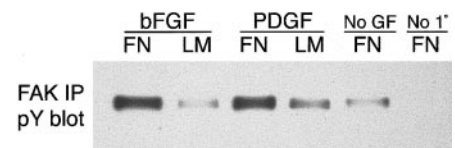

FIG. 5. Activation of FAK by growth factors in PCASMCs cultured on FN and LM. PCASMCs were cultured for 2 h on dishes that had been precoated with either 20 μg/ml FN or 20 μg/ml LM (as indicated). PDGF-BB (1 ng/ml), bFGF (5 ng/ml) or no growth factor (No GF lane) were then added for 10 min, cell lysates were collected and FAK was immunoprecipitated with mAb 2A7. Note that one sample (No 1° lane) received no anti-FAK antibody to serve as a control. Immunoprecipitated proteins were then analyzed by immunoblotting with anti-phosphotyrosine mAb 4G10 to determine the amount of tyrosine phosphorylated FAK in each sample.

(19). In some systems it appears that ERK1/2 activation is downstream of FAK activation, while in other systems ERK1/2 activation can occur in the absence of FAK activation (19, 34, 35). PCASMCs do not appear to require FAK activation for ERK1/2 activation because cells on LM show robust ERK1/2 activation but no FAK activation in response to growth factors. Signaling through Shc has been shown to play an important role in ERK1/2 activation in response to integrin ligation in endothelial cells (35). This activation of ERK1/2 through Shc is independent of FAK activation, and is therefore reminiscent of our results with PCASMCs on FN and LM. One difference between endothelial cells and SMCs is that LM does not support ERK1/2 activation in endothelial cells but it does in PCASMCs. The likely explanation for this is the repertoire of LM-binding integrins expressed by these cells types. Endothelial cells rely on $\alpha 2\beta 1$ and $\alpha 6\beta 1$ for binding to LM (integrins that do not activate Shc $\rightarrow$ ERK signaling), whereas SMCs interact with LM through $\alpha 1\beta 1$ (an integrin that does activate Shc $\rightarrow$ ERK signaling), $\alpha 2\beta 1$ and $\alpha 3\beta 1$ (7, 19, 35). Thus, our findings are consistent with previous results showing the effects of different integrins on ERK1/2 activation.

Our results can help explain why SMCs in the media typically do not proliferate in response to serum factors, while cells in the intima of atherosclerotic plaques do proliferate. SMCs in the media are surrounded by basement membranes which contain laminin but lack FN, whereas cells in the intima of atherosclerotic plaques are surrounded by a matrix that is high in FN. Our data suggest that cells surrounded by a basement membrane (i.e., high LM, low FN) would not proliferate and would express high levels of smooth muscle myosin, even in the presence of serum growth factors. In contrast cells surrounded by a matrix rich in FN are poised to proliferate in response to serum growth factors. We now know that integrin signaling pathways, and in particular signals through FAK, play an important role in this differential response to growth factors. Thus, FAK may be an important target in future therapies aimed at reducing SMC proliferation and migration in diseases such as atherosclerosis and post-angioplasty restenosis.

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