Activation of the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ and focal adhesion kinase (FAK) during arteriogenesis

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Abstract Migration and proliferation of smooth muscle cells (SMC) are important events during arteriogenesis, but the underlying mechanism is still only partially understood. The present study investigates the expression of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ as well as focal adhesion kinase (FAK) and phosphorylated FAK (pY397), key mediators for cell migration and proliferation, in collateral vessels (CV) in rabbit hind limbs induced by femoral ligation or an arteriovenous (AV) shunt created between the distal femoral artery stump and the accompanying femoral vein by confocal immunofluorescence. In addition, the effect of the extracellular matrix components fibronectin (FN), laminin (LN), and Matrigel on expression of these focal adhesion molecules proliferation was studied in cultured SMCs. We found that: (1) in normal vessels (NV), both integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ were mainly expressed in endothelial cells, very weak in smooth muscle cells (SMC); (2) in CVs, both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ were significantly upregulated ($P < 0.05$); this was more evident in the shunt-side CVs, 1.5 and 1.3 times higher than that in the ligation side, respectively; (3) FAK and FAK(py397) were expressed in NVs and CVs in a similar profile as was $\alpha_5\beta_1$ and $\alpha_v\beta_3$; (4) in vitro SMCs cultured on fibronectin (overexpressed in collaterals) expressed higher levels of FAK, FAK (pY397), $\alpha_5\beta_1$, and $\alpha_v\beta_3$ than on laminin, whereas SMCs growing inside Matrigel expressed little of these proteins and showed no proliferation. In conclusion, our data demonstrate for the first time that the integrin-FAK signaling axis is activated in collateral vessels and that altered expression of FN and LN may play a crucial role in mediating the integrin-FAK signaling pathway activation. These findings explain a large part of the positive remodeling that collateral vessels undergo under the influence of high fluid shear stress.

Keywords Arteriogenesis · Integrins · Focal adhesion kinase · Extracellular matrix

Introduction

Arteriogenesis is a remodeling process of pre-existing small arteriolar vessels into larger collateral vessels. One main feature of collateral vessel growth is the neointima formation. We previously reported that the cellular mechanism involved in neointima formation includes active extracellular proteolysis, extracellular matrix remodeling, smooth muscle cell proliferation, migration, and phenotype changes [1–3]. Among these events, extracellular proteolysis paves the way for smooth muscle migration, decreased laminin is in favor of dedifferentiation of contractile smooth muscle cells, whereas increased fibronectin provides the trace for smooth muscle migration and also facilitates smooth muscle proliferation. However, the outside-in or inside-out signal transduction events associated
with cell migration occurring during collateral vessel growth remain to be determined.

Integrins are a family of transmembrane receptors consisting of an alpha and a beta chain that are the principle mediators of cell interactions with the extracellular matrix [4]. Integrin-ECM interactions play an important role in a diverse variety of important biological processes [5]. The integrin \( \alpha_5\beta_1 \) receptor mediates cell adhesion and migration by recognition of fibronectin, and provides proliferative signals to vascular cells [6]. The vitronectin receptors (VnR) v3 and v5 have been implicated in the migration of a variety of cell types including smooth muscle [7] and endothelial cells [8]. Furthermore, integrins \( \alpha_5\beta_1 \) and \( \alpha v\beta 3 \) have been shown to be involved in atherosclerosis, restenosis after angioplasty, and constrictive vascular remodeling after injury [9]. Vascular injury induces \( \alpha 5\beta 1 \) integrin expression exclusively in proliferating VSMCs at the luminal surface of the neointima [10], and VSMC invasion from the tunica media to the intima has been shown to be dependent on \( \alpha v\beta 3 \) integrin expression [11].

The nonreceptor tyrosin kinase focal adhesion kinase (FAK) is a cytoplasmic protein that localizes to focal contacts and adhesions which link to the extracellular matrix. Its N-terminal FERM domain is important for signal integration from growth factor receptors. Its C-terminal FAT region contains binding sites for integrin-associated proteins such as paxillin and talin. FAK is activated by integrin clustering, also by various mechanical stimuli and soluble factors, and is considered as a key signal component at focal adhesions. FAK is expressed in most tissues and cell types including vascular cells. Recently, FAK was reported to be involved in blood vessel morphogenesis and to regulate smooth muscle cell proliferation and phenotype [12, 13].

Based on the information cited above, we hypothesized that the FAK-integrin axis was activated during collateral vessel growth. To test this hypothesis, we first used immunofocal microscopy with specific antibodies to determine the expression of FAK, FAK (pY397), and integrins \( \alpha 5\beta 1 \) and \( \alpha v\beta 3 \) in collateral vessels in rabbit hind limb induced by femoral ligation or by an arteriovenous shunt created unilaterally between the distal femoral artery stump and the accompanying femoral vein. The simply ligated contralateral side was used as control. Then the skin was closed with sterile surgical clips. The animals were allowed to recover completely, and housed with free access to water and food. We did not observe any gangrene or gross impairment of hind limb function after femoral artery occlusion and arteriovenous fistula creation. All animals received antibiotic (Bencylpenicillin) and analgesic treatment (Buprenorphin).

### Tissue sampling

At day 7 post-surgery, the animals were re-anesthetized, the collateral vessel tissues were removed and immediately frozen in liquid nitrogen, embedded in tissue processing medium (O.C.T.), and stored at \(-80^\circ\text{C} \) till further use. In addition, the femoral artery and its branches from normal rabbits without either ligation or shunt were used in this study. The animals were anesthetized with an i.m. injection of midazolam (1 mg/kg) and xylazine (5 mg/kg), and acute bilateral femoral artery ligation was performed with two knots. Following immediate occlusion, an arteriovenous (AV) shunt was created unilaterally side-to-side, between the distal femoral artery stump and the accompanying femoral vein. The simply ligated contralateral side was used as control. Then the skin was closed with sterile surgical clips. The animals were allowed to recover completely, and housed with free access to water and food. We did not observe any gangrene or gross impairment of hind limb function after femoral artery occlusion and arteriovenous fistula creation. All animals received antibiotic (Bencylpenicillin) and analgesic treatment (Buprenorphin).

### Immunohistochemistry

Cryosections were cut 5-\( \mu \text{m} \) thick and fixed in 4% paraformaldehyde. For integrins \( \alpha 5\beta 1 \) and \( \alpha v\beta 3 \) staining acetone fixation was used, and then pre-incubated in 0.2% BSA-C (Aurion Co.) and thereafter incubated with the...
primary antibodies (Table 1). Incubation of secondary antibodies (Donkey anti-mouse-IgG or anti-goat-IgG, Dianova, Germany, at 1:200) was followed by Cy2 or Cy3 conjugated Streptavidin (Biotrend). The nuclei were stained with 7-aminoactinomycin D or TOTO3 (Molecular Probes). The sections were coverslipped and viewed with a Leica confocal microscope (Leica TCS SP). Further documentation and image analysis were carried out using a Silicon Graphics Octane workstation (Silicon Graphics) and three-dimensional multichannel image processing software (Bitplane).

Immunostaining for cultured cells was performed following a similar protocol as described above, except for primary antibody incubation which was conducted at 4°C overnight.

Cell culture

We used primary cultures of rabbit aortic smooth muscle cells (SMCs), which were isolated and cultivated as described previously for porcine aortic SMCs [14]. Cells were cultivated routinely in medium 199 (PAA; Coelbe, Germany) with 20% FCS; when they reached confluence they were split 1:4. Cells between passage four and six were used for the described experiments.

Culture of SMC on extracellular matrix material (ECM)

Plastic 12-well culture plates (Greiner; Frickendorf, Germany) containing 18-mm round glass coverslips were coated with either 5 μg/cm² laminin (BD Biosciences) or 5 μg/cm² fibronectin (BD Biosciences), for 1 h at room temperature. Remaining material was aspirated and the plates were rinsed carefully. SMCs were seeded on top of the protein layer and incubated at 37°C, 5% CO₂ until they were 50–75% confluent. For cell culture in a three-dimensional matrix, SMCs were added to 150 μl/cm² Matrigel Basement Membrane Matrix (BD Biosciences) as instructed by the manufacturer. Plates were incubated for 30 min at 37°C and cell culture medium was added.

Cell proliferation in vitro

Cell proliferation of smooth muscle cells cultured on different substrates, FN, LN, and Matrigel Basement Membrane Matrix was detected with 5-Bromo–2′-deoxyuridine Labeling and Detection Kit 1 (Roche Diagnostics GmbH, Germany) according to the manufacturer’s protocol.

In all staining procedures, incubation with PBS instead of the first antibody was used as negative control to exclude nonspecific binding of the secondary detection system.

Quantitative measurements

The quantification of immunofluorescence intensity was performed with a Leica TCS SP confocal microscope, using the quantitation software from Leica as described previously [3]. Briefly, one channel with format 512 and appropriate filters was used. A full range of gray values from black to peak white (0-pixel to 255-pixel intensity level) was set during the whole process of measurements. The intensity of fluorescence was expressed as arbitrary units AU/μm².

Quantitation of BrdU-positive cells was performed with the confocal microscope. The counting was done at 40×, and the ratio of BrdU-positive nuclei to all nuclei of vascular wall cells or smooth muscle cells was considered as proliferation index.

All data are presented as mean ± SEM. ANOVA test was used to examine the difference between normal vessels and growing vessels.

Results

Expression of FAK and FAK(pY397) in vivo and in vitro

In normal arterial vessels, endothelial cells expressed FAK, but the majority of the media SMCs and adventitial cells only weakly expressed FAK. FAK(pY397) was mainly detected in endothelial cells. One week after surgery FAK and FAK(pY397) were significantly up-regulated in both ligation and shunt-side collateral vessels. The increase in shunt-side was more evident, being 1.3-fold and 1.5 fold over that in only ligation side (Fig. 1a–c, Table 2). The immunostaining of FAK and FAK(pY397) in serial sections revealed that increased expression of FAK was accompanied by an increase of expression of FAK(pY397) Fig. 1d–e).

We tested the possible role of the components of the basement membrane in regulating the expression of FAK and FAK (pY397) in smooth muscle cells. We found that

### Table 1  Primary and secondary antibodies used in this study

| Antigen     | Clone | Host  | Dilution | Company          |
|-------------|-------|-------|----------|------------------|
| Integrin αvβ3 | LM609 | Mouse | 1:100    | Chemicon         |
| Integrin α5β1 | IBS5  | Mouse | 1:100    | Chemicon         |
| FAK         | 77    | Mouse | 1:100    | BD Transduction  |
| FAK(pY397)  | 14    | Mouse | 1:100    | BD Transduction  |
| Anti-mouse-IgG |  Donkey | 1:100 |          | Dianova, Germany |
smooth muscle cells cultured on fibronectin substrate highly expressed FAK and FAK (pY397), to a lesser amount on Laminin substrate and very little inside a Matrigel basement membrane matrix (Fig. 2a–f, Table 3).

Expression of integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in vivo and in vitro

In normal arterial vessels, integrin $\alpha_5\beta_1$ was faintly present in all vascular cells but $\alpha_v\beta_3$ was only expressed in endothelial cells. In growing collateral vessels, both

Table 2 Quantitative analysis of immunofluorescence density (AU/μm²) of FAK, FAK(pY397), and integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in normal (NV), control (only ligation) (CLV), and AV-shunt vessels (1 week)

|          | NV ($N = 12$) | CLV ($N = 16$) | AV-shunt (19) |
|----------|---------------|----------------|---------------|
| FAK      | 66.31 ± 8.14  | 128.26 ± 8.30 | 166.32 ± 9.90* |
| FAK(pY397)| 24.86 ± 4.11  | 54.09 ± 7.13  | 81.28 ± 3.74* |
| $\alpha_5\beta_1$ | 43.15 ± 4.27  | 93.0 ± 7.93   | 136.75 ± 10.05* |
| $\alpha_v\beta_3$ | 23.53 ± 3.41  | 80.08 ± 4.83  | 105.28 ± 9.59* |

1-way ANOVA test  
* $P < 0.05$ versus CLV or NV

Fig. 1 Confocal micrographs of FAK and FAK(pY397) immunostaining in normal (NV), control (only ligation) (CLV), and AV-shunt (AV-S) collateral vessels. a–d: FAK; e: FAK(pY397); a: NV; b: CLV, c, d and e: AV-S. Note that AV-S showed strong staining for both FAK and FAK(pY397), d and e showing colocalization of FAK and FAK(pY397) in serial sections

Table 2

|          | NV ($N = 12$) | CLV ($N = 16$) | AV-shunt (19) |
|----------|---------------|----------------|---------------|
| FAK      | 66.31 ± 8.14  | 128.26 ± 8.30 | 166.32 ± 9.90* |
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1-way ANOVA test  
* $P < 0.05$ versus CLV or NV

Fig. 2 Confocal micrographs of FAK and FAK(pY397) immunostaining in smooth muscle cells cultured on different substances. F-actin was stained by Rodamin-labelled phalloidin. a–c: for FAK; d–f: for FAK(pY397); a and d: on fibronectin; b and e: on laminin; c and f: inside Matrigel basement membrane matrix. Note that fibronectin was the strongest stimulator of expression of FAK and FAK(pY397)
proteins were upregulated in the vascular wall. A more marked expression was seen in shunt-side collateral vessels (Fig. 3a–f). Integrin $\alpha_5\beta_1$ was 1.5 times higher and $\alpha_v\beta_3$ 1.3 times higher in shunt-side collateral vessels than in the ligation side (Table 3). In vitro experiments with smooth muscle cells cultured on fibronectin substrate showed strong staining for both integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, while on laminin substrate, smooth muscle cells expressed less integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. Inside the Matrigel basement membrane matrix, smooth muscle cells expressed little integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Fig. 4, Table 3).

Profile of cell proliferation in vitro

A BrdU labeling test was performed in vitro in this study. The index of cell proliferation in smooth muscle cells on fibronectin, laminin, and inside the Matrigel basement membrane was 38%, 28%, and 0% (Fig. 5a–c), respectively.

### Table 3

Quantitative analysis of immunofluorescence density (AU/\(\mu m^2\)) of FAK, FAK(py397), and integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ in SMC cultured on different substances, fibronectin (FN), laminin (LN), Matrigel and without substance (C)

|        | FN      | LN     | Matrigel |
|--------|---------|--------|----------|
| FAK    | 139.96 ± 14.68* | 86.39 ± 9.66 | 35.48 ± 9.51 |
| FAK(py397) | 74.81 ± 8.05*  | 40.97 ± 4.94  | 18.78 ± 8.06  |
| $\alpha_5\beta_1$ | 65.65 ± 7.86* | 33.92 ± 5.08  | 6.95 ± 2.20   |
| $\alpha_v\beta_3$ | 45.91 ± 4.06*  | 30.03 ± 2.91  | 8.57 ± 4.23   |

1-way ANOVA test
*P < 0.05 versus LN or Matrigel

Discussion

Smooth muscle cells in the media are enveloped by a basement membrane mainly composed of collagen IV and laminin, preventing SMC from proliferation and migration and keeping SMC in a contractile state [15–17]. We demonstrated previously that active extracellular proteolysis is present in the process of arteriogenesis, which includes increased expression of MMP-2 and -9, degradation of the basement membrane and smooth muscle cell migration resulting in neointima formation [2]. We also found that fibronectin is upregulated during arteriogenesis [1]. In this study the molecular mechanisms by which the extracellular matrix and intracellular signaling regulate SMC migration were examined by analyzing the expression of focal adhesion kinase (FAK), and integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. The expression of phosphorylated FAK(py397), a marker for FAK activation, was also detected. The phosphorylation at Tyr397 creates a high affinity binding site for the Src homology 2 domain of Src family kinases [18, 19]. We found all these proteins upregulated during arteriogenesis, and much more marked in shunt-side collateral vessels, which indicates that the FAK-integrin signaling axis was activated in the process of collateral vessel growth.

The studies describing impairment of migration in FAK-deficient cells and its restoration by the reconstitution with wild-type FAK [20] implicate that FAK plays an important role in cell migration. Furthermore, intimal hyperplasia correlated with overexpression of FAK in smooth muscle cells [21], whereas overexpression of FRNK, an endogenous...
inhibitor of FAK, inhibits migration and proliferation of smooth muscle cells [22]. Taken these findings together, our data suggest that upregulation of FAK during arteriogenesis may be crucial for smooth muscle cell migration and neo-intima formation.

FAK can mediate cell motility and migration via inside-out signaling mechanisms. Inhibition of FAK expression led to inhibition of MMP-9 gene expression [23], whereas MMP inhibitors augment fibroblast adhesion through stabilization of focal adhesion contacts [24]. It has been suggested that FAK activation in an in vivo environment may synchronize MMP-mediated extracellular proteolysis and cell motility [23]. Fibronectin is an important component of the extracellular matrix to facilitate cell migration. A recent study shows that FAK promotes organization of the fibronectin matrix and fibrillar adhesions [25]. In growing collateral vessels, upregulation of MMPs, fibronectin, and FAK have been observed, therefore we hypothesize that smooth muscle cell migration is partly regulated in arteriogenesis through a FAK inside-out signaling mechanism.

Integrin α5β1 is mainly a fibronectin-receptor. It mediates most of fibronectin’s biological activities, of which, integrin α5β1 triggering by soluble or anchored fibronectin promotes cellular locomotion [26]. Its weak expression in the media of normal arteries of rabbit hind limb is consistent with the small amount of fibronectin present in the basement membrane, indicating that this receptor does not play an important role in regulating the function of smooth muscle cells in normal vessels. The induction and upregulation of integrin α5β1 observed after vascular injury, in atherosclerosis and in ductus closure, suggest that it may be involved in smooth muscle cell migration and proliferation, contributing to blood vessel repair [10, 27]. Our novel finding of dramatic upregulation of integrin α5β1 suggests that this integrin plays an important role in collateral vessel growth. The correlation of upregulation of integrin α5β1 with increased expression of fibronectin [1], extracellular proteolysis, neointima formation [2] and proliferation is in accordance with the notion that integrin α5β1 is a factor for cell migration and proliferation which is confirmed by the in vitro experiments of this study.
Integrin αvβ3 is mainly a vitronectin-receptor, but various other ligands have been documented. This receptor is not present in adult smooth muscle cells, but is induced in the neointima, media, and adventitia after vascular injury [28], whereas the αvβ3 inhibiting RGD peptide, G-Pen-GRGDSPCA, or XI 735 (a selective cyclic Arg-Gly-Asp (RGD) limit neointimal hyperplasia and lumen stenosis [28, 29]. Recently, Choi et al. showed that this receptor mediates intimal smooth muscle accumulation in the neointima after carotid ligature in mice [30]. These studies indicate that integrin αvβ3 plays an important role in regulating smooth muscle cell migration and proliferation. Currently, the role of integrin αvβ3 in arteriogenesis is not clear. Since integrin αvβ3 facilitating cell invasion [31], it mediates SMC migration by stimulating SMC-MMP production [32] and it regulates α5β1-mediated cell migration toward fibronectin [33], we suppose that the upregulated integrin αvβ3 may be an important contributor to smooth muscle cell migration and neointima formation.

Factors mediating expression of FAK and integrins 5β1 and αvβ3 in vascular cells currently remain to be determined. However, increasing body of evidence suggests the underlying mechanism for these protein’s expression is probably associated with increased blood flow, upregulation of growth factors and inflammation present in collateral vessel. Firstly, shear stress and high intraluminal pressure were reported to activate integrins and FAK and α5β3 up-regulate expression of FAK [34–36]. Secondly, both FGF2 and PDGF BB increased integrin 5β1 expression in cultured smooth muscle cells [10]. Furthermore FGF increased expression of integrins αvβ3 in collateral vessels [37]. VEGF activated integrin 5β1 [38], and the synergism between their receptors and integrins in angiogenesis was also observed recently [39]. Thirdly, Stimulation of smooth muscle cells with inflammatory cytokines increased integrin 5β1 expression [40]. The present study adds to the knowledge that that changes in extracellular matrix components may be also responsible for regulation of expression of FAK and integrins 5β1 and αvβ3 during arteriogenesis, which will be discussed below.

FAK connects to growth factor receptors through FAK FERM domain and to integrins through C-terminal domain. It integrates growth-factor and integrin signals to promote cell migration. Integrin clustering upon binding to extracellular matrix components, such as FN, or growth factor binding to their receptors results in activation of FAK [41]. Therefore, upregulation of growth factors and the integrins α5β1 and αvβ3 indicates an increase in “outside-in” signaling during arteriogenesis. Different integrins on cells interact with different ECM components; for example, smooth muscle cells adhere to FN mainly through the integrin α5β1, while they adhere to laminin through α1β1, α2β1, and α3β1. Because of the mechanism by which FAK is activated and the fact that FN and the integrin α5β1 are present in the media of normal arterial vessels at a very low level, but highly upregulated in growing collateral vessels, we hypothesize that the changes in ECM components, such as increase in fibronectin and decrease in laminin and collagen IV may be involved in the production of the integrins α5β1 and αvβ3 as well as of FAK and FAK(pY397) during collateral vessel growth. Our data demonstrate that cells cultured on fibronectin proliferate at a higher rate and express high level of the integrins α5β1 and αvβ3 as well as of FAK and FAK(pY397), whereas cells cultured on laminin proliferate at a low rate and express lower levels of integrins and FAK and FAK(pY397). This is more evident in cells growing inside Matrigel (a basement membrane protein preparation which contains laminin and collagen IV). Here, cells do not proliferate and express only very low levels of integrins and FAK and FAK(pY397) indicating that extracellular matrix components can differentially regulate expression of integrins, FAK and FAK(pY397) as well as smooth muscle cell proliferation. The effects of fibronectin and laminin on cell proliferation and activation of FAK are consistent with previous reports. Morla et al. found cells growing on FN had a higher proliferation rate than those on LN. Hedin et al. found cells cultured on LN produced few focal adhesions, and tyrosine phosphorylation of proteins was less than in cells on FN [12, 42]. To our knowledge, we present here the first report using three-dimensional basement membrane Matrigel to study cell-matrix adhesions.

Our findings from in vitro experiments can explain at least in part the mechanism by which the integrins α5β1 and αvβ3, FAK, and FAK(pY397) are present at a lower level and smooth muscle cells do not proliferate in normal arterial vessels, while they are upregulated and smooth muscle cells have a higher rate of proliferation during collateral vessel growth. In normal arterial vessels, smooth muscle cells are surrounded by a layer of basement membrane which is rich in laminin and collagen IV, but lacks fibronectin. Therefore, smooth muscle cells express few integrins and FAK and FAK(pY397) and not proliferate. In growing collateral vessels, laminin and collagen IV undergo degradation, while fibronectin is induced in large amounts, resulting in upregulation of the integrins α5β1 and αvβ3 and in activation of FAK and smooth muscle cell proliferation.

Taken together, our data contribute new information to the mechanism for arteriogenesis by showing activation of the integrin-FAK signaling axis in collateral vessels. This activation, at least in part, is mediated by extracellular matrix components facilitating smooth muscle cell proliferation and migration.

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