Overexpression of the focal adhesion kinase ($p125^{FAK}$) in the vascular smooth muscle cells of intimal hyperplasia

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Purpose: **The migration and proliferation of vascular smooth muscle cells (VSMCs) are important events in the development of intimal hyperplasia (IH). The focal adhesion kinase (FAK) gene encodes a protein tyrosine kinase ($p125^{FAK}$) involved in signal transduction pathways used in cell adhesion, motility, and proliferation. Because alterations in these cellular processes are thought to occur in VSMCs during IH, we studied FAK expression in healthy arteries and veins in comparison with that in pathologic vessels containing IH.**

Methods: **To determine $p125^{FAK}$ expression at the cellular level, we developed a monoclonal antibody that specifically detected FAK in formalin-fixed, paraffin-embedded tissue sections (5 μm) and analyzed the levels of FAK expression in human arteries and veins. Specificity of monoclonal antibody 4.47 was demonstrated by means of immunofluorescence microscopy showing FAK-specific staining at focal adhesions of healthy human vascular smooth muscle cells (AoSMCs). By using immunohistochemistry techniques, we analyzed the expression of $p125^{FAK}$ in 25 adult human vascular tissue samples from individual patients, which contained a histologically confirmed healthy artery, vein, or IH.**

Results: **FAK expression in healthy and pathologic human vascular tissue was localized predominantly within VSMC cytoplasm. In healthy human artery and vein, borderline FAK expression was detected in the media of seven of 17 vessels and undetectable in the remainder of specimens. However, in vessels containing IH, FAK was overexpressed in the pathologic VSMC populations at moderate-to-strong levels in eight of eight specimens. The levels of FAK expression were directly correlated with structures containing IH, and the results of FAK staining intensity and the percentage of positive cells in these samples were significantly increased compared with normal vascular tissue levels ($P < .05$, Student *t* test).**

Conclusion: **These results provide the first evidence that FAK is overexpressed in VSMCs involved in IH and suggest that FAK upregulation may be part of a mechanism for migration and proliferation of VSMCs during this process. Furthermore, the dramatic upregulation of FAK in IH and the relative lack of expression in healthy vessels suggest that FAK may be a rational target for controlling IH. (J Vasc Surg 2001;34:344-9.)**

The long-term success of bypass grafting and angioplasty for the treatment of atherosclerotic occlusive disease is limited by the development of intimal hyperplasia (IH), the most common cause of reconstructive failure.[1] IH is a complex process occurring in the vessel wall after endothelial cell injury. Vascular smooth muscle cell (VSMC) migration and proliferation and extracellular matrix deposition leads to intimal thickening, luminal narrowing, and eventual vessel thrombosis. Efforts to better understand the molecular genetics involved in IH have included many studies that examine the role of inflammatory cells, growth factors, extracellular matrix proteins, and nitric oxide. Nevertheless, despite advances, a clear understanding of the molecular events and signal transduction pathways responsible for the development of IH has yet to be established.

A novel nonreceptor tyrosine kinase known as the focal adhesion kinase (FAK) is a critical mediator of signaling events between cells and their extracellular matrix. This 125-kD protein ($p125^{FAK}$) is ubiquitously expressed in most tissues and is localized to discrete regions along ventral cell surfaces known as focal adhesions where integrin receptors interact with extracellular matrix proteins of the underlying substratum.[2-4] FAK is the major tyrosine-phosphorylated protein in focal adhesions and has been shown to interact with integrins,[5] other tyrosine kinases (Src, Fyn),[6] and growth factors (VEGF, PDGF).[7,8] FAK has been linked to cancer and appears to be involved in the proliferative changes when human tumors progress from benign to invasive phenotypes.[9-12] In tumor cells, FAK is thought to have a dual function, both promoting tumor cell adhesion and acting as a survival signal to inhibit apoptosis as a tumor develops anchorage-independent growth properties.[13] Thus, in both healthy and neoplastic cells, FAK appears to be a key element in signal transduction pathways used in cell adhesion,[14] motility,[15] and apoptosis.[16-18] Because similar alterations in these cellular processes occur in VSMCs during IH,[19,20] we studied

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FAK expression in a variety of healthy and abnormal human vascular tissues.

METHODS

Primary tissues and cell lines. Twenty-five adult human vascular tissue samples were obtained from operative specimens banked through protocols approved by the Institutional Review Board at the University of North Carolina (UNC) Hospitals. Histopathologic tissue confirmation was performed on all specimens by a reference pathologist (M.V.I.), and hematoxylin and eosin-stained sections were reviewed, with areas of healthy artery, vein, and IH identified. The samples containing IH were from preocclusive vein graft stenoses obtained at the time of primary graft revision. The vascular tissues were sharply dissected from adjacent tissues, and samples were formalin-fixed, paraffin-embedded (FFPE) and stored by the UNC-Lineberger Comprehensive Cancer Center Tissue Procurement and Analysis Facility. Human cell lines were obtained from Clonetics (San Diego, Calif).

Immunofluorescence microscopy. Human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (AoSMCs; 5×10^4) were plated onto cover slips in 6-well tissue culture dishes and fixed in 3.7% formaldehyde. After permeabilization in 0.1% Triton X-100, the cells were blocked with 10% normal goat serum and incubated with anti-FAK (4.47) monoclonal antibody (1:50 dilution), an antibody that our group had developed earlier (available from Upstate Biotechnology, Lake Placid, NY).[21] After incubation with the secondary antibody conjugated with fluoresceine isothiocyanate (Molecular Probes, Eugene, Ore), positive cells were visualized and photographed with a Zeiss fluorescence microscope. The cells were co-stained with antipaxillin monoclonal antibody (Transduction Laboratory, Lexington, Ky) with rhodomamine conjugated secondary antibody to determine the specific localization of $p125^{FAK}$. Paxillin is a focal adhesion protein.

Immunohistochemistry assays. Five-micron thick vascular tissue sections were cut from corresponding FFPE blocks and subsequently deparaffinized and hydrated by passing through xylene and a graded series of ethanol concentrations (100%-70%). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide, and mercuric pigments were removed by incubating sections in Auto/Iodine (Fischer Scientific) for 1 minute. Slides were incubated in Redusol (Fischer Scientific), a nonspecific blocking agent, two times for 2 minutes. The hydration process was completed by rinsing three times for 3 minutes in 1× Automation Buffer (Fischer Scientific).

Heat-induced epitope recovery was used by submerging the sections in 1× Antigen Retrieval Citra Buffer (Biogenex) and steam heating in a standard steamer (Black and Decker) for 30 minutes. After blocking in normal horse serum for 15 minutes, the sections were incubated with anti-FAK 4.47 monoclonal antibody (1:50 dilution, 0.3 μg) overnight at 4°C. Control sections were incubated with a comparable concentration (1:37, 0.3 μg) of isotype-matched IgG_1 monoclonal antibody, MOPC-21 (Sigma, St Louis, Mo), and a VSMC marker and α-actin monoclonal antibody (1:100, 0.15 μg; Sigma). After extensive washings in automation buffer, the sections were incubated with biotinylated horse anti-mouse IgG, followed by avidin peroxidase with the Vectastatin ABC elite kit (Vector Laboratories, Burlingame, Calif). The chromogenic reaction was performed with 3-3' diaminobenzidine (DAB), toned with the DAB enhancing solution

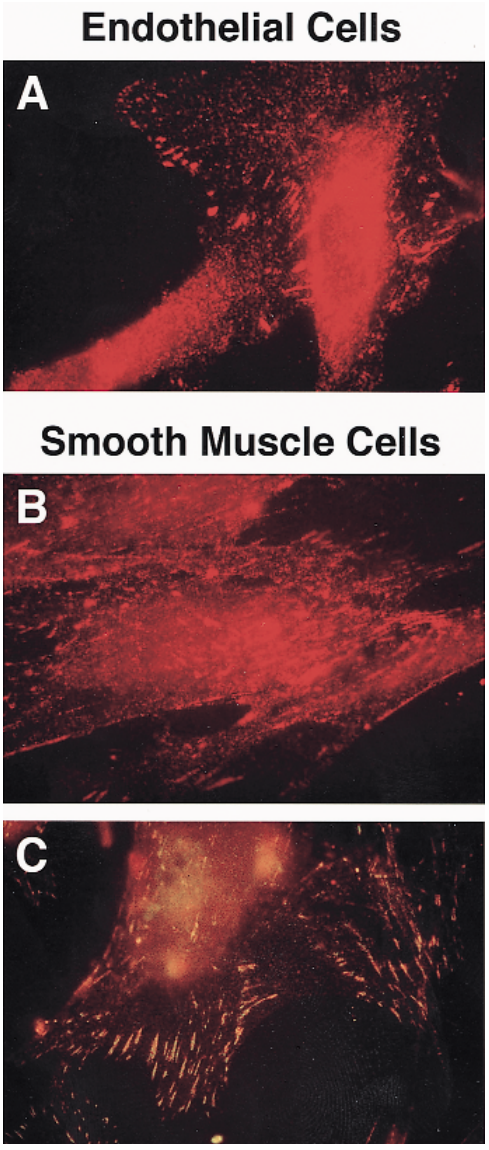

Fig 1. Immunofluorescence microscopy of $p125^{FAK}$ expression in human vascular endothelial and smooth muscle cells. Indirect immunofluorescence demonstrates focal adhesion kinase staining at focal adhesions. **A,** Human aortic endothelial cells. **B,** Human aortic smooth muscle cells. **C,** Dual immunofluorescence staining demonstrates that focal adhesion kinase (*red*) and paxillin (*green*) co-localize at focal adhesions (*yellow*) in human aortic smooth muscle cells.

Immunohistochemical analyses of $p125^{FAK}$ expression in healthy artery, vein, and intimal hyperplasia

Patient	*Tissue type**	*Staining intensity*	*% positive cells*
1	Vein	+	5
2	Vein	0	0
3	Vein	0	0
4	Vein	+	5
5	Vein	+	5
6	Vein	0	0
7	Vein	0	0
8	Vein	0	0
9	Vein	+	<5
10	Vein	+	<5
11	Vein	0	0
12	Vein	0	0
13	Artery	+	5
14	Artery	0	0
15	Artery	+	5
16	Artery	0	0
17	Artery	0	0
18	IH	+++	30
19	IH	+++	60
20	IH	+++	80
21	IH	++++	90
22	IH	++++	80
23	IH	+++	40
24	IH	+++	40
25	IH	++++	60

*Histologically confirmed tissue.
Vein, Healthy vein media, vascular smooth muscle cells; *Artery,* healthy artery media, vascular smooth muscle cells; *IH,* intimal hyperplasia, vascular smooth muscle cells.

(Vector Laboratories). The sections were counterstained with hematoxylin, washed in 1× automation buffer, and dehydrated in 100% ethanol and xylene.

Immunohistochemistry scoring. Each tissue section was scored for FAK expression by a single pathologist blinded to the source. Smooth muscle cells from each section were scored for staining intensity on the basis of this scoring system: 0, none; 1+, borderline; 2+, weak; 3+, moderate; 4+, strong. Staining characteristics of cellular localization (cytoplasm, nucleus, membrane, or a combination thereof) and overall distribution (homogeneous throughout cell population, unifocal, heterogeneous, or multifocal) were recorded.

Statistics. The statistical differences between staining intensity and the percentage of positive cells in healthy vascular tissue and IH were evaluated by means of the Student *t* test at an overall .05 level. Values are expressed as the mean ± SE.

RESULTS

Immunofluorescence microscopy of $p125^{FAK}$ expression in human vascular endothelial and smooth muscle cells. Indirect immunofluorescence microscopy was performed on HAECs and AoSMCs to determine the presence of $p125^{FAK}$ expression at the cellular level in cells isolated from healthy human vascular tissue. Our anti-FAK monoclonal antibody (4.47) localized $p125^{FAK}$ specifically to the focal adhesions of these cells (Fig 1). Furthermore, dual-staining of FAK and paxillin, a focal adhesion protein known to associate with FAK,[14] demonstrated that these two proteins colocalized at focal adhesion sites.

Overexpression of $p125^{FAK}$ in intimal hyperplasia. To characterize FAK expression in vascular disease, we extended our studies to human tissues performing immunohistochemistry (Table). In all specimens demonstrating FAK expression (healthy and IH), FAK was localized predominately within the cytoplasm of VSMCs, and the levels of $p125^{FAK}$ expression were heterogeneous in pathologic specimens containing smooth muscle cell populations involved in IH. The presence of VSMCs contained within the IH of the samples studied was confirmed on adjacent sections after immunohistochemical staining for α-actin, a VSMC marker.

In the 17 samples of healthy vascular tissue obtained from patients encompassing healthy veins and arteries, there was little detectable $p125^{FAK}$ expression within the VSMCs (Fig 2). Only five samples of venous tissue (from patients 1, 4, 5, 9, and 10) demonstrated any FAK immunoreactivity, with only minimal (1+) expression in 5% of the VSMCs comprising the media of these samples. Likewise, FAK levels in the smooth muscle cells contained within the media of healthy human artery was low, with only two samples (patients 13 and 15) showing any detectable expression (1+) in 5% of the cells.

In contrast, $p125^{FAK}$ was detected at high levels in all eight of the IH specimens we tested. The significant upregulation of FAK expression in these IH specimens is demonstrated in the near-occlusive venous bypass graft

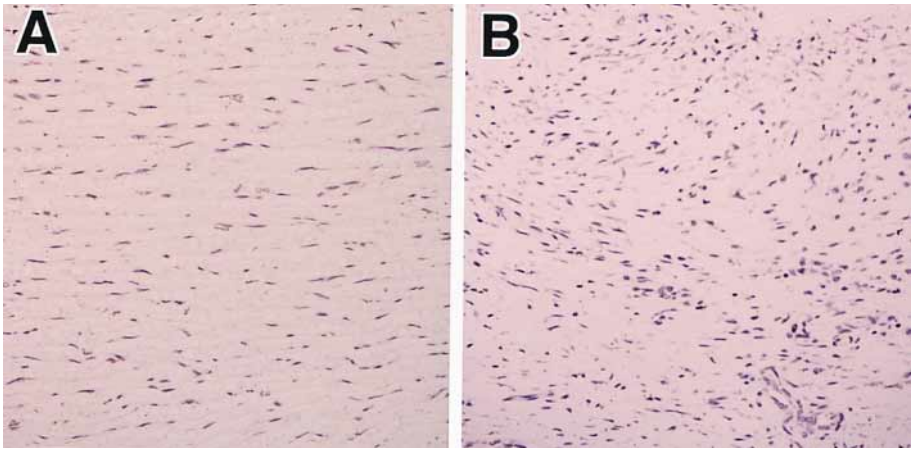

Fig 2. Expression of $p125^{FAK}$ in healthy human artery and vein (400×). No detectable FAK expression is observed in vascular smooth muscle cells comprising media of healthy human artery **(A)** and vein **(B)**.

lesion from patient 21 (Fig 3). This sample contained a failing infrainguinal autogenous vein bypass graft. Here, the occlusive process of IH containing the pathologic VSMCs demonstrated strong (4+) FAK expression throughout the cytoplasm of most (95%) of the VSMCs.

The intensity of FAK expression in IH VSMCs ranged from moderate (3+) to strong (4+) in the smooth muscle cells (mean = 3.4 ± 0.5) from the pathologic populations involved in IH. Smooth muscle cells from adjacent histologically normal areas demonstrated minimal FAK expression. However, the entire population of VSMCs was not involved in the intimal hyperplastic specimens overexpressed $p125^{FAK}$. As noted in the Table, there was a variability in the percentage of VSMCs demonstrating positive FAK staining, ranging from 30% to 90% (mean, 60 ± 22) of smooth muscle cells (Fig 4). The results from staining intensity and the percentage of positive cells in IH were significantly different from the expression levels found in healthy tissue ($P < .05$, Student t test). Because the vascular tissue specimens studied were from isolated early (healthy tissue) and late stages (near-occlusive lesions) of the intimal hyperplastic process, a conclusion about the timing of FAK expression in the vessel wall during this process remains unclear.

DISCUSSION

These results provide the first evidence that the capacity of VSMCs to undergo IH is accompanied by the overexpression of $p125^{FAK}$. From our data, we conclude that VSMCs contained within healthy arteries and veins are not accompanied by aberrant $p125^{FAK}$ expression. In contrast, significantly high levels of FAK expression were present in VSMCs contained within primary near-occlusive lesions obtained from failing bypass grafts. The smooth muscle cells in these intimal lesions had migrated from the media across the external elastic lamina into the intima, creating a hyperplastic process. Taken together, these results suggest that the FAK may be a part of a signaling pathway in VSMCs that promotes IH.

It is intriguing to speculate why $p125^{FAK}$ might become overexpressed in VSMCs as part of the intimal hyperplastic process. FAK is a normal gene with detectable levels in all tissues including blood vessels[2,22] and no evidence for mutations, which would render it a transforming gene.[23] In healthy cells, FAK is a critical mediator of signaling events between cells and their extracellular matrix while being linked to cell adhesion,[14] motility,[15] and apoptosis.[16-18,24] The ongoing events observed in the early development of IH appear to be explained by specific hemodynamic factors and inflammatory cell pathways.[1] However, although the mechanisms are not well understood, the migration and proliferation of VSMCs may be later events in IH that require additional molecular changes.[25] Smooth muscle cells may develop further signaling imbalances that lead to alterations in cell adhesion and motility, allowing VSMCs to migrate through the external elastic lamina and potentially develop IH.

A phenomenon similar to this has been shown to occur in cancer. High levels of FAK expression were observed in human invasive and metastatic tumors as tumor cells became invasive and migrated through the extracellular matrix.[4-7] Furthermore, FAK has been shown to be a survival signal for cells that grow independent of matrix signaling, and the downregulation of FAK function in tumor cells, which grow in an anchorage-independent manner, has been shown to result in apoptosis.[13] Thus, if similar events occur during the process of IH, it is conceivable that VSMCs upregulate FAK expression to acquire the ability to migrate, proliferate, and survive within the intimal area of an injured vessel.

Finally, our observations raise the possibility that FAK might be a rational therapeutic target to interrupt the intimal hyperplastic process. Because we have reproducibly demonstrated minimally detectable $p125^{FAK}$ levels in

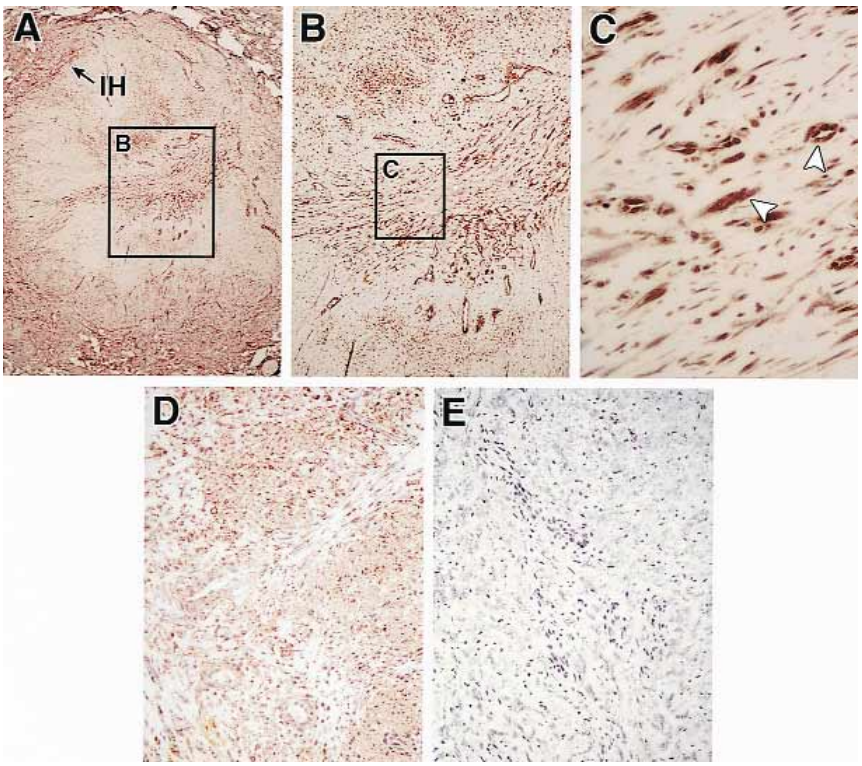

Fig 3. p125^{FAK} overexpression in intimal hyperplasia. Cross section of segment of infrainguinal autogenous vein bypass graft containing intimal hyperplasia. Near-occlusive tissue contains migrating vascular smooth muscle cells and extracellular matrix material involved in proliferation of intimal hyperplasia. *Arrow* marks the edge of the vessel wall. **A,** Cross section of venous bypass graft demonstrating intense, diffuse immunoreactivity for p125^{FAK} (200×). **B,** Higher magnification demonstrating p125^{FAK} overexpression in vascular smooth muscle cells (400×). **C,** Highest magnification demonstrating p125^{FAK} overexpression contained within cytoplasm of vascular smooth muscle cells (600×). **D,** Cross section of venous bypass graft demonstrating diffuse immunoreactivity for α-actin (400×). **E,** Cross section of venous bypass graft after immunohistochemical staining for isotype-matched IgG_1 (400×). *IH,* Intimal hyperplasia.

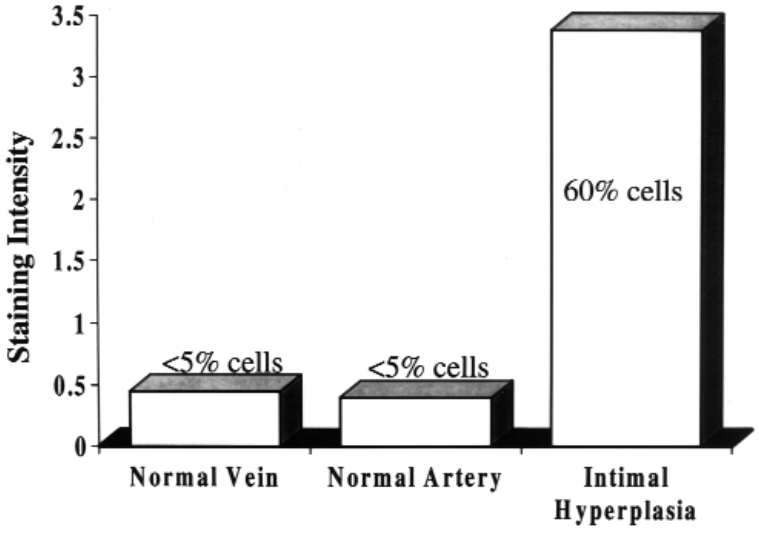

Fig 4. Graphical representation of p125^{FAK} expression levels in healthy vein and artery, compared with vessels containing intimal hyperplasia. Staining intensity (0 to 4+) is represented on y-axis. Also indicted is focal adhesion kinase expression distribution (% positive staining).

healthy vascular tissue and high levels of $p125^{FAK}$ in IH, it would be useful to study the effects of attenuating FAK expression in VSMCs involved in IH. If this causes disruption of the intimal hyperplastic process, molecular-based therapeutics against FAK may become useful in the treatment of IH.

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