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Wirkungen der Gerinnungsfaktoren Thrombin und Faktor-Xa auf vaskuläre Zellen des Menschen

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1 Einleitung

1.1 Pathophysiologische Bedeutung des Gerinnungssystems

Gefäßwand, Thrombozyten und die Proteine des Gerinnungssystems wirken bei der Blutstillung funktionell so zusammen, dass bei einer Verletzung ein Gefäß möglichst rasch abgedichtet wird, um einen übermäßigen Blutverlust zu verhindern. Gleichzeitig werden gerinnungshemmende Mechanismen aktiv, um eine überschießende Hämostase und damit einen drohenden thrombotischen Verschluss eines Gefäßes zu vermeiden. Die klassische Theorie der Entstehung von Thrombosen wurde durch den Pathologen Rudolf Virchow (1821-1902) begründet (143). Demnach wirken auf die Entstehung von Thrombosen im Wesentlichen drei Faktoren ein: 1. Veränderungen an der Gefäßwand, 2. Veränderungen der Strömungsgeschwindigkeit des Blutes, 3. Veränderungen in der Zusammensetzung des Blutes (Virchow-Trias) (69). Später wurde dieses Konzept durch die Begriffe primäre und sekundäre Blutstillung ergänzt (2, 129). Dabei wird die Adhäsion von Thrombozyten an der verletzten Gefäßwand, gefolgt von einer Thrombozytenaktivierung und Aggregatbildung, als primäre Hämostase bezeichnet. Die nachfolgende Aktivierung des Gerinnungssystems, welche letztlich zur Spaltung von Fibrinogen und Polymerisierung von Fibrin führt, wird sekundäre Hämostase genannt (Abb.1).

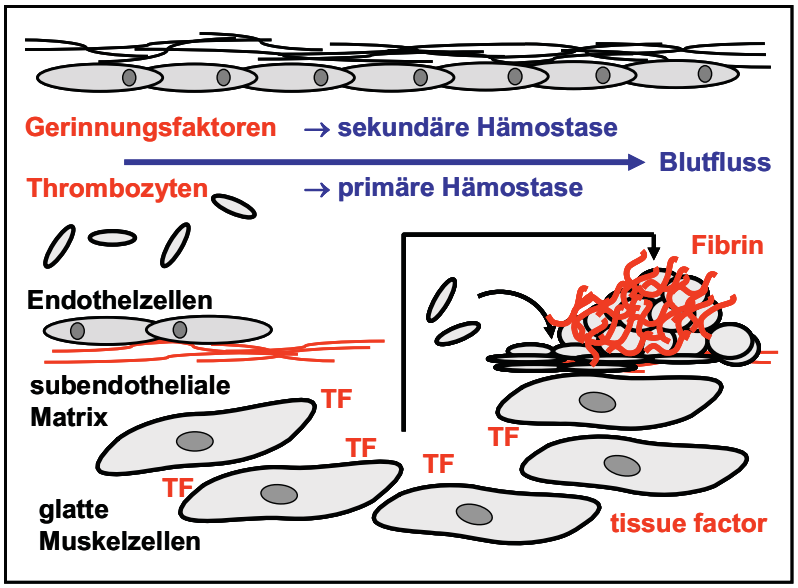

Abb.1:

Thrombozyten haften an einer Verletzung an um eine rasche Blutstillung zu bewirken (primäre Hämostase). Kontakt des Blutes mit in der Gefäßwand vorliegendem tissue factor (TF) initiiert die Blutgerinnung. Das gebildete Fibrin stabilisiert den Thrombus (sekundäre Hämostase) (modifiziert nach K. Schrör, 2005) (116).

Die Thrombozyten binden über spezifische Rezeptoren, die Glykoprotein (GP) IIb/IIIa Rezeptoren, an Fibrin, wodurch der initial instabile Thrombus stabilisiert wird. Nach der heutigen Vorstellung des Ablaufes der Blutgerinnung sind primäre und sekundäre Hämostase nicht zwei zeitlich aufeinander folgende Abläufe, sondern greifen ineinander und laufen gleichzeitig ab, dabei kommt den Zellen der Gefäßwand und den Thrombozyten eine besondere Bedeutung zu (47). Abbildung 2 stellt die derzeitige Vorstellung vom Ablauf der Blutgerinnung schematisch dar.

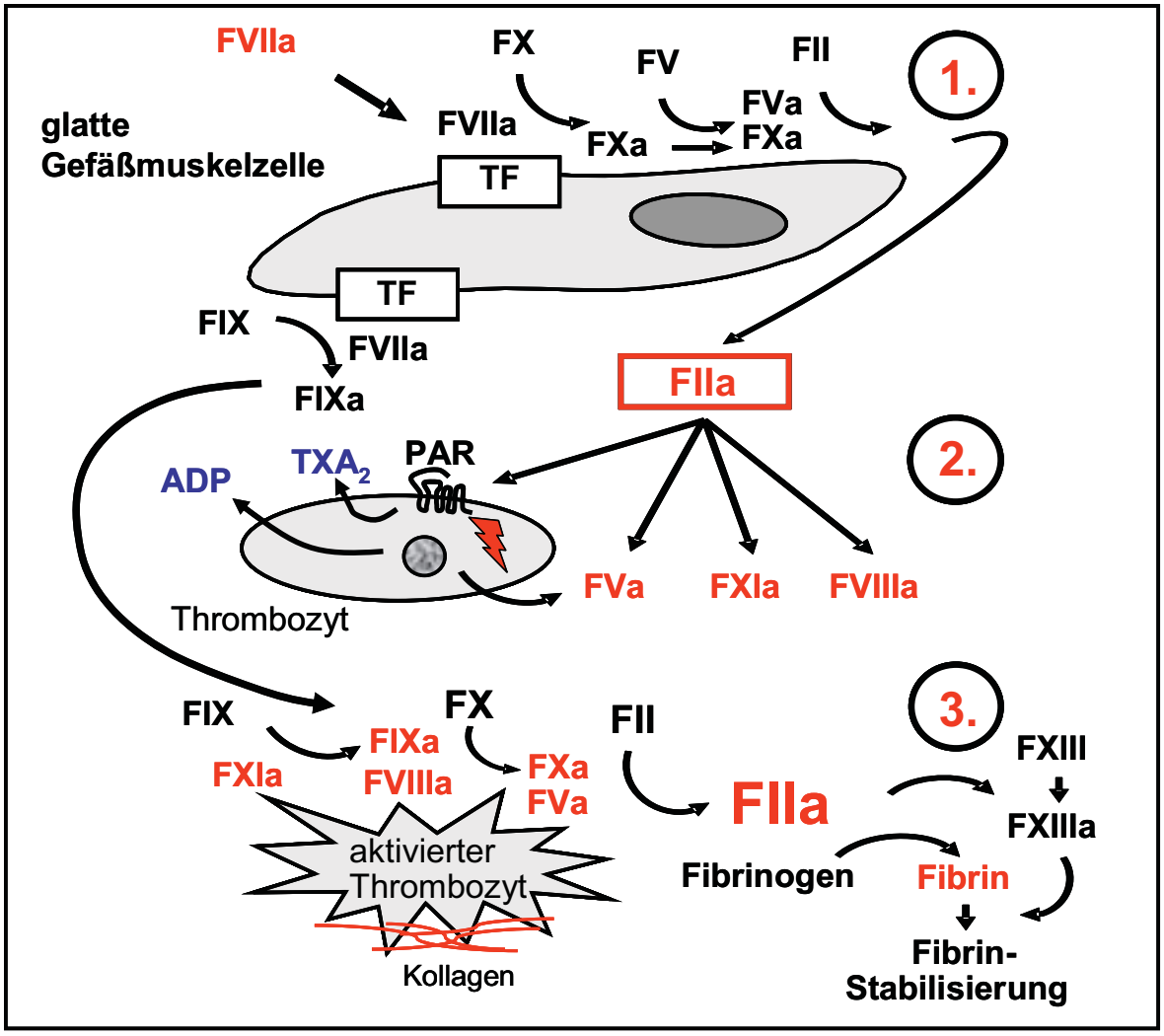

Abb. 2: Das Model der „zellabhängigen“ Blutgerinnung. **1.** Bedingt durch eine Gefäßverletzung kommt es zur Freisetzung von Kalzium und Phospholipiden aus zerstörten Zellen der Gefäßwand. Hierdurch aktivierter Faktor-VII (FVIIa) bindet an TF auf der Membran von Gefäßmuskelzellen und Fibroblasten. Der an TF gebundene FVIIa aktiviert FX (FXa), dessen Aktivität durch FVa verstärkt wird. Dieser sog. Prothrombinasekomplex spalte Prothrombin (FII). **2.** Die initial geringen Mengen des gebildeten Thrombin (FIIa) bewirken eine Aktivierung der an der Verletzungsstelle an Kollagen gebundenen Thrombozyten über Protease-aktivierte Rezeptoren (PAR) sowie die Aktivierung weiterer Gerinnungsenzyme (FVa, FXa, FVIIIa). Es kommt zur Ausschüttung von Mediatoren, welche die Thrombozytenaktivierung verstärken (ADP, TXA_2), und von gespeicherten Gerinnungsfaktoren (FV). **3.** Durch an der Oberfläche von aktivierten Thrombozyten akkumulierte Gerinnungsfaktoren (FIXa/VIIIa, FXa/Va) kommt es zur raschen Bildung großer Mengen Thrombin, welche die Spaltung von Fibrinogen und die Fibrinpolymerisierung bewirken. Die Aktivierung von FXIII (FXIIIa) stabilisiert das Fibringerinnsel (modifiziert nach Weber et al., 2006) (147).

Initiiert wird die Blutgerinnung nach Gefäßverletzung durch Bindung von aktiviertem Faktor-VII (FVIIa) an tissue factor (TF) auf der Oberfläche von glatten Gefäßmuskelzellen und Fibroblasten der Adventitia (29, 104). Dies führt zur Bildung von aktiviertem Faktor-X (FXa), welcher wiederum Thrombin aus Prothrombin bildet. Bereits geringe Mengen von Thrombin führen zur Aktivierung von Blutplättchen, welche durch Bindung von Gerinnungsfaktoren an ihrer Oberfläche eine vermehrte Bildung von Thrombin bewirken (s. Abb. 2). Thrombin ermöglicht durch Abspaltung der Fibrinopeptide A und B vom Fibrinogenmolekül dessen Polymerisierung, was schließlich zur Ausbildung eines Thrombus an der Gefäßwand führt (77, 128). Interessant und bislang nicht völlig verstanden ist die Beobachtung, dass Thrombin nicht nur in Mengen gebildet wird, die zur Bildung eines Thrombus erforderlich sind. Bereits 5% des in einem Thrombus gebildeten Thrombin genügen zur effizienten Initiierung der Fibrinbildung (72). Hieraus, und aus dem Vorhandensein von spezifischen Rezeptoren auf Zellen der Gefäßwand, welche durch Thrombin aktiviert werden können – den Protease-aktivierten Rezeptoren (PARs) – lässt sich ableiten, dass es Thrombin-vermittelte zelluläre Wirkungen gibt, die über die Spaltung von Fibrin hinausgehen (46, 110, 130).

1.2 Wirkungen von Gerinnungsfaktoren auf die Gefäßwand

Nach Ausbildung eines Thrombus kommt es zur Aktivierung von Proteasen, beispielsweise von Plasmin, welche eine Fibrinolyse und damit die Auflösung des Thrombus bewirken (129). Zusätzlich erfolgt eine Freisetzung von Signalmolekülen aus dem Thrombus, z.B. dem Thromboxan A_2 (TXA_2) aus Blutplättchen oder verschiedener Wachstumsfaktoren und Zytokine, welche proliferative und entzündliche Reaktionen in den Zellen der Gefäßwand bewirken (2, 20, 127, 148). Obwohl das Ziel dieser Mechanismen letztlich die Wiederherstellung der Gefäßkontinuität ist, können dieselben Prozesse zur Pathogenese von Gefäßerkrankungen wie der Atherosklerose, die seit längerem als entzündliche Erkrankung verstanden wird, beitragen (66, 78, 107, 127, 131). Eine wichtige Bedeutung für die Wirkungen des Gerinnungssystems auf die Gefäßwand geht dabei von Thrombin und FXa aus. Neben ihrer Eigenschaft als Gerinnungsfaktoren stimulieren sie die Proliferation und Migration glatter Gefäßmuskelzellen (13, 95,

123). Proliferation und Migration glatter Gefäßmuskelzellen stellen Schlüsselereignisse für die Entwicklung der Atherosklerose und Restenose nach Gefäßverletzung dar (107, 122). Somit können Thrombin und FXa zur Pathogenese dieser Erkrankungen beitragen (127). Insbesondere konnten wir in unseren Arbeiten zeigen, dass FXa hierbei eigenständige Effekte, also unabhängig von seiner Thrombin-aktivierenden Wirkung, auf die Zellen der Gefäßwand ausüben kann. Die Effekte von Thrombin und FXa in vaskulären Zellen werden durch Protease-aktivierte Rezeptoren (PARs) vermittelt (15, 16), welche eine Untergruppe der G-Protein-gekoppelten Rezeptoren (GPCRs) darstellen (70, 86). Eine Übersicht der Wirkungen eines wandständigen Thrombus auf die Gefäßwand ist in Abbildung 3 dargestellt.

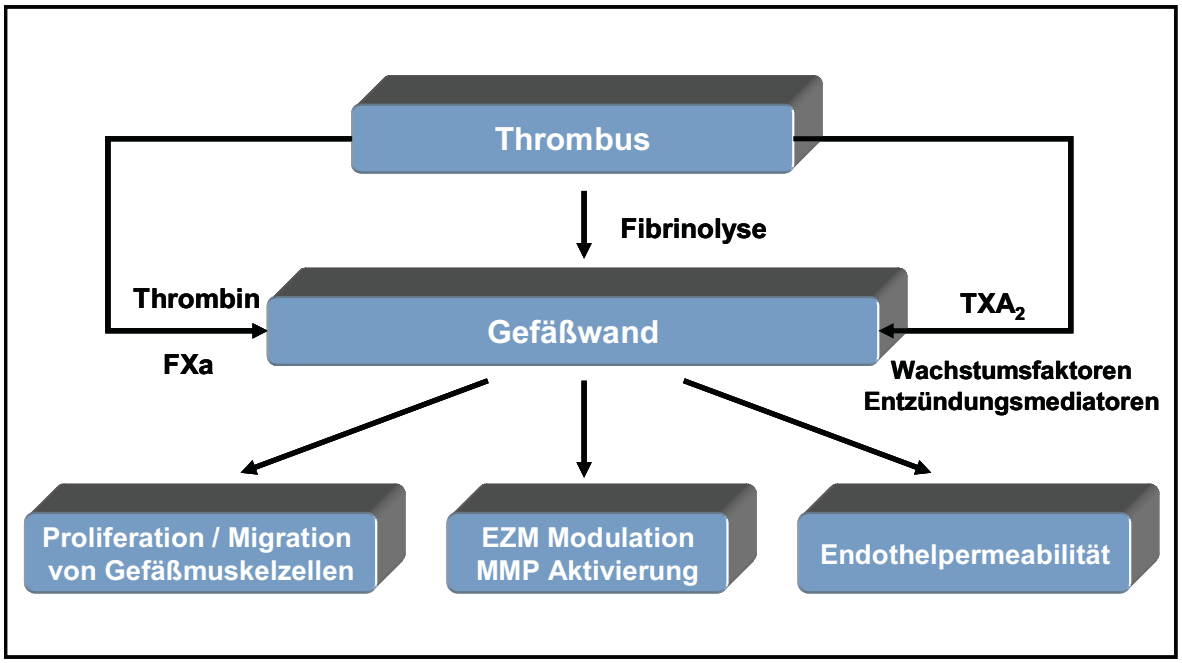

Abb. 3: Effekte eines Thrombus auf die Gefäßwand. Nach Ausbildung eines Thrombus kommt es zur Fibrinolyse. Von Blutplättchen gebildetes Thromboxan (TXA_2), freigesetzte Wachstumsfaktoren und Entzündungsmediatoren wirken auf die Gefäßwand ein. Ebenso können Thrombin und Faktor-Xa (FXa) - unabhängig von seiner Thrombin-aktivierenden Funktion - auf Zellen der Gefäßwand einwirken. Sie beeinflussen die Proliferation und Migration glatter Gefäßmuskelzellen, modulieren die Struktur der extrazellulären Matrix (EZM), indem sie Matrixmetalloproteinasen (MMPs) aktivieren, und regulieren darüber hinaus die Permeabilität des Gefäßendothels.

Die zellulären Wirkungen von Thrombin und FXa für Proliferation, Mitogenese und Migration humaner Gefäßmuskelzellen und somit mögliche molekulare Mechanismen, über die diese Gerinnungsfaktoren an der Pathogenese von Gefäßerkrankungen beteiligt sein können, wurden in mehreren Arbeiten, welche

teils auch Grundlage dieser Habilitationsschrift sind, in unserer Arbeitsgruppe untersucht (13, 16, 94-97).

1.3 Protease-aktivierte Rezeptoren

Protease-aktivierte Rezeptoren (PARs) sind G-Protein-gekoppelte Rezeptoren, die durch proteolytische Abspaltung des extrazellulären Endes des Rezeptors aktiviert werden (Abb. 4). Die dabei entstehende neue N-terminale Aminosäuresequenz funktioniert als endogener Ligand, taucht in den Rezeptor ein und (auto-)aktiviert diesen (16, 70, 86). Mittlerweile wurden vier PARs beschrieben, wobei PAR-1, -3 und -4 durch Thrombin, PAR-2 durch FXa oder durch andere Serinproteasen wie Trypsin aktiviert werden (16, 70, 86). Abbildung 4 stellt einige der durch PAR-1 regulierten Signalwege stark vereinfacht dar.

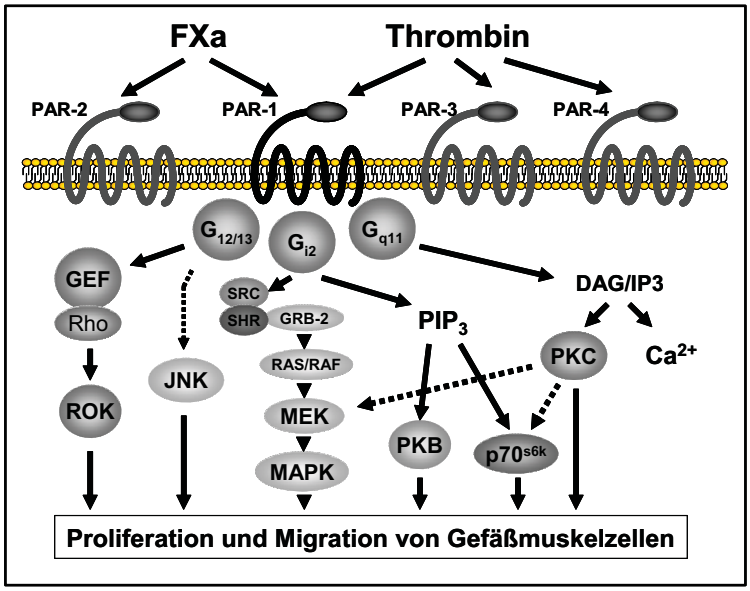

Abb. 4: Aktivierung und mitogene Signalwege Protease-aktivierter Rezeptoren (PARs). Thrombin aktiviert PAR-1, -3 und -4, FXa PAR-1 und -2. Dargestellt sind einige durch PAR-1 stimulierte Signalwege. Abkürzungen: DAG, diacylglycerol; SRC, pp60src-related kinases; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PKB/PKC, protein kinase B/C; p70s6k, p70-S6 kinase; GEF, Rho GTP exchange factor; ROK, Rho-activated kinase; PIP_3, phosphatidylinositol 3,4,5-tris-phosphate, IP3, Inositoltri-phosphate; (modifiziert nach Macfarlane, 2001) (70).

Der „klassische“ Thrombinrezeptor PAR-1 wurde 1991 kloniert (146). Er ist ein ubiquitär vorkommender Rezeptor. In Thrombozyten stellt seine Aktivierung das stärkste Signal der Plättchenaktivierung dar. PAR-1 wird in Endothelzellen, Gefäßmuskelzellen und Fibroblasten exprimiert. Er besitzt wichtige Funktionen im ZNS und ist in fast allen Tumorarten vorhanden (70, 86). Über PAR-3 und -4 ist weniger bekannt. In Arbeiten aus unserem Labor wurde kürzlich die Bedeutung von PAR-3 und -4 für das Wachstum humaner Gefäßmuskelzellen und ihre mögliche Beteiligung an der Entstehung von Gefäßerkrankungen beschrieben (14,

16). Über die Bedeutung des PAR-2 ist ebenfalls wenig bekannt. Auch dieser Rezeptor wird bei pathologischen Veränderungen des Gefäßes, beispielsweise im Rahmen der Atherosklerose (73) und des Diabetes (8, 108), vermehrt gebildet und scheint somit eine Bedeutung für das Krankheitsgeschehen zu besitzen. Über die Mechanismen, welche die Expression der PARs regulieren und somit für die Beteiligung dieser Rezeptoren in verschiedenen Krankheitsbildern relevant sind, ist bislang wenig bekannt. Die Regulation des PAR-1 ist Bestandteil der hier dargestellten Arbeiten und auch Ziel zukünftiger Untersuchungen.

1.4 Bedeutung der extrazellulären Matrix für zelluläre Wirkungen von Gerinnungsfaktoren

Der Begriff extrazelluläre Matrix (EZM) beschreibt die Substanz, welche Zellen umgibt, also den Interzellularraum ausfüllt. Sie stellt im Wesentlichen ein Gemisch aus Proteinen, Proteoglykanen, Glykoproteinen und Kohlenhydraten dar (11, 17, 92). Die EZM unterliegt einer ständigen Erneuerung. Veränderungen ihrer Zusammensetzung sind an der Pathophysiologie von Gefäßerkrankungen, z.B. der Atherosklerose und Restenose nach Gefäßverletzung, beteiligt (17, 92). Dabei kommt der EZM nicht nur eine passive Funktion als Stützgerüst für die Zellen der Gefäßwand zu, sondern ihre Komponenten beeinflussen den Phänotyp der Zellen, indem sie z.B. Proliferation und Zellmigration fördern können (92). Die EZM kann auf verschiedene Weisen modifiziert werden. Entsprechende Stimuli, beispielsweise Wachstumsfaktoren oder Entzündungsmediatoren, können die Produktion von EZM-Bestandteilen durch die Zellen der Gefäßwand verändern oder es können Mechanismen aktiviert werden, welche die vorhandene EZM modulieren. Eine Familie von extrazellulären Proteasen, welche Moleküle der EZM, z.B. Kollagen, abbauen, sind die Matrixmetalloproteinasen (MMPs) (52, 67, 126, 142). In eigenen Studien wurde die Wirkung von Thrombin und FXa auf MMPs untersucht (Arbeit #1) (94). Im Mittelpunkt standen hier die MMP-2 und MMP-9, welche aufgrund ihrer Eigenschaft, Gelatine zu verdauen, auch als Gelatinase A und B bezeichnet werden (89). Die Bedeutung der MMP-2 scheint in der Förderung von Proliferation und Migration glatter Muskelzellen zu liegen (140). In einer neueren Arbeit wurde auch eine Beteiligung an der Entwicklung der

Atherosklerose beschrieben (63). MMP-9 ist ebenfalls an der Entstehung zahlreicher Erkrankungen beteiligt. Es wurde eine Bedeutung für Entzündungsprozesse, Atherosklerose und Plaqueruptur, sowie Angiogenese und Tumorwachstum beschrieben (89).

Ein Bestandteil der EZM ist die Hyaloronsäure (HA), welche ein Gemisch aus langkettigen Zuckermolekülen ist (133, 149). HA wird im Rahmen von atherosklerotischen Gefäßveränderungen verstärkt gebildet (133, 149). Die Regulation von HA-Synthese durch Wachstumsfaktoren und seine Funktion für die Migration von Zellen deuten auf eine wichtige Bedeutung bei Gefäßerkrankungen hin (115). Dass Thrombin über eine Induktion der HA-synthetisierenden Enzyme, den Hyaluronsäuresynthasen, an der Regulation der HA beteiligt ist, wurde kürzlich in unserer Arbeitsgruppe gezeigt (Arbeit #2) (141).

1.5 Pharmakologische Beeinflussung des Gerinnungssystems

Gerinnungshemmende Pharmaka besitzen eine breite Anwendung in der Klinik. Allein in Deutschland betrug die Verordnung von Vitamin-K-Antagonisten (Phenprocoumon und Warfarin) im Jahr 2005 204,1 Mio. Tagesdosen, die der Heparine (niedermolekulare und unfraktionierte) 64,9 Mio. Tagesdosen (121). Da insbesondere unter der Therapie mit Vitamin-K-Antagonisten ein hohes Risiko gefährlicher Blutungen besteht, ist das Bestreben groß, alternative Substanzen zu finden, die bei gleichem Schutz vor Thrombosen ein geringeres Blutungsrisiko aufweisen (116, 117).

Interessante Ziele für die Entwicklung neuer Antikoagulanzien sind die beiden Hauptenzyme der Gerinnungskaskade, Thrombin und FXa (38). Spezifische Inhibitoren dieser beiden Proteasen werden derzeit von mehreren großen Pharmakonzernen entwickelt. Der Vorteil dieser Inhibitoren besteht zum einen in der möglichen oralen Applikation (117). Im Gegensatz zu den Heparinen, welche nach oraler Einnahme nicht in den Organismus aufgenommen, sondern im Darm verdaut werden, stellen die neueren Thrombin- bzw. FXa-Inhibitoren niedermolekulare Substanzen dar, die aus dem Darm aufgenommen werden. Ein weiterer Vorteil solcher kleiner Moleküle besteht in einer möglichen besseren Wirkung auf ein bereits bestehendes Blutgerinnsel. Während Heparin als großes

Molekül nicht innerhalb des Klots wirken kann, können die kleineren Moleküle in den Klot gelangen und hier eine Lyse des Klots begünstigen. Aus demselben Grund könnten die direkten Thrombin- und FXa-Inhibitoren auch eine stärkere Wirkung auf das Zellwachstum und damit die Restenoserate nach Gefäßverletzung zeigen (4), als dies für Heparine der Falls ist (114). Da das Heparin nicht an den Ort der Wirkung von Thrombin und FXa - die Interaktionsstelle des Gerinnsels mit der Gefäßwand bzw. innerhalb der Gefäßwand - gelangt, findet sich bei Patienten keine hemmende Wirkung von Heparinen auf das Wachstum von Gefäßmuskelzellen (41, 114), obwohl seine proliferations- und migrationshemmende Wirkung auf diese Zellen *in vitro* und in Tiermodellen auch *in vivo* seit langem gut belegt ist (23, 24, 43).

Im Gegensatz zum Heparin, welches Thrombin und FXa in Gegenwart von Antithrombin hemmt, bewirken Phenprocoumon und Warfarin über eine Inhibition der γ-Carboxylase, welche Vitamin-K als Substrat benötigt, eine Hemmung der Vitamin-K-abhängig in der Leber synthetisierten Gerinnungsfaktoren (FII, FVII, FIX, FX). Hierbei kommt es durch mangelnde Carboxylierung zur Bildung nicht funktionaler Gerinnungsfaktoren (134). Die Einstellung der Patienten ist aufgrund der langen Halbwertzeiten der Medikamente und der geringen therapeutischen Breite schwierig und bedarf einer kontinuierlichen Überwachung, um dem Risiko gefährlicher Blutungen vorzubeugen. In Studien, welche die Wirkung von Vitamin-K-Antagonisten auf die Restenoserate nach Ballondilatation (PTCA, perkutane transluminale coronare Angioplastie) bei Patienten mit koronarer Herzkrankheit (KHK) untersuchten, zeigte sich nur dann eine hemmende Wirkung, wenn die Patienten ausreichend lange vorbehandelt wurden (135). Eine im Anschluss an die PTCA begonnene Behandlung mit Vitamin-K-Antagonisten zeigt keinen Erfolg (41), was sich durch die Dauer von mehreren Tagen bis zur Wirksamkeit des Medikamentes erklären lässt. Dies könnte auf eine Beteiligung von Thrombin bzw. FXa an der Ausbildung einer Restenose beim Patienten hindeuten. Allerdings ist eine Vorbehandlung mit Vitamin-K-Antagonisten vor PTCA in der Praxis nicht anwendbar, da das Blutungsrisiko unvertretbar wäre und ein großer Prozentsatz der Ballondehnungen als Notfallmaßnahme durchgeführt wird. Eine längerfristige Vorbehandlung der Patienten ist somit meist nicht möglich. Es bleibt abzuwarten, ob die derzeit entwickelten direkten Thrombin- und FXa-Inhibitoren hier Abhilfe bringen.

2 Zusammenfassende Darstellung der Ergebnisse und Schlussfolgerungen

Ziel der hier dargestellten Untersuchungen war es, molekulare Wirkungen der Gerinnungsfaktoren Thrombin und FXa an Zellen humaner Blutgefäße zu untersuchen, über die diese beiden Faktoren zur Pathogenese der Atherosklerose beitragen können. Im Vordergrund stand dabei die Erforschung von Mechanismen, die an der Proliferation und Migration glatter Gefäßmuskelzellen beteiligt sind.

2.1. Effekte von Thrombin und FXa auf die extrazelluläre Matrix

Veränderungen der EZM stellen einen wichtigen pathogenetischen Faktor für die Entstehung von Gefäßerkrankungen dar (11). Im Rahmen der Atherosklerose kommt es zu einem Abbau von Kollagen und von elastischen Fasern in der EZM, was zur Verhärtung der Gefäßwand beiträgt (56, 124). Induktion von Matrix-abbauenden Enzymen, wie den MMPs, kann zu einem Aufbrechen einer athosklerotischen Plaque führen mit Exposition von Matrixmolekülen, z.B. Kollagen, gegenüber dem Blutstrom (39). An einer sollten „offenen Verletzung“ im Gefäß kommt es zur Adhäsion von Thrombozyten und - bei einer kompletten Thrombose des Gefäßes – zu einem Infarkt distal des Verschlusses. Neben ihrer Funktion als Gerinnungsfaktoren können Thrombin und FXa an der Modulation der EZM mitwirken. Dies kann über die Induktion der Synthese von EZM-Bestandteilen durch Gefäßmuskelzellen (45) oder durch Aktivierung EZM-abbauender Enzymen - den MMPs - bedingt sein (40). In eigenen Arbeiten wurden die Wirkungen von Thrombin und FXa auf MMP-2 und MMP-9 sowie die EZM-Modulation untersucht (94, 141).

2.1.1 Aktivierung der MMP-2 durch FXa

Arbeit #1 Rauch BH, Bretschneider E, Braun M, Schrör K. Factor Xa releases matrix metalloproteinase-2 (MMP-2) from human vascular smooth muscle cells and stimulates the conversion of pro–MMP-2 to MMP-2: Role of MMP-2 in Factor Xa–induced DNA synthesis and matrix invasion. *Circ Res* 2002; 90:1122-1127.

Matrixmetalloproteinasen (MMPs) stellen eine Familie strukturell verwandter Zink-Endopeptidasen dar. Sie sind am Umsatz von EZM-Bestandteilen beteiligt, aber auch an physiologischen Vorgängen in der Embryonalentwicklung, Gewebereparatur und Angiogenese, sowie an pathophysiologischen Prozessen, wie der Entwicklung von Atherosklerose, Entzündungen und Tumorerkrankungen (1, 81, 89). MMPs werden als inaktive Pro-Enzyme von Zellen nach extrazellulär sezerniert und können dort durch bereits aktivierte MMPs oder zellmembranständig durch den Urokinase-typ Plasminogenaktivator (uPA)/Plasmin Komplex aktiviert werden (89). Ein weiterer Weg speziell der MMP-2-Aktivierung stellt die Freisetzung von membrangebunderer MMP-2 aus dem Komplex mit MT1-MMP (membran-typ-1 MMP) und dem endogenen Inhibitor TIMP-2 (tissue-inhibitor of metalloproteinases-2) dar (49, 50).

Aufgrund der Bedeutung der MMP-2 für Proliferation und Migration glatter Gefäßmuskelzellen (140) und der Beobachtung, dass Thrombin MMP-2 in Gefäßmuskelzellen aktiviert wird (40), haben wir in der **Arbeit #1** untersucht, ob FXa Wirkungen auf die MMP-2 ausübt. In kultivierten Gefäßmuskelzellen, die aus humanen V. saphena-Explantaten isoliert wurden, konnte gezeigt werden, dass FXa konzentrations- und zeitabhängig eine Überführung der Pro-Form des Enzyms in die aktive MMP-2 bewirkt. Weitere mechanistische Untersuchungen deuteten darauf hin, dass es nicht nur zu einer direkten proteolytischen Spaltung und damit Aktivierung des Proenzyms kommt, sondern dass FXa aktive MMP-2 von der Zelloberfläche freisetzen kann. Es wurde eine Freisetzung von auf der Zellmembran an MT1-MMP/TIMP-2 gebundener MMP-2 durch FXa angenommen. Die möglichen Aktivierungswege von MMP-2 durch hämostatische Proteasen sind in Abbildung 5 dargestellt.

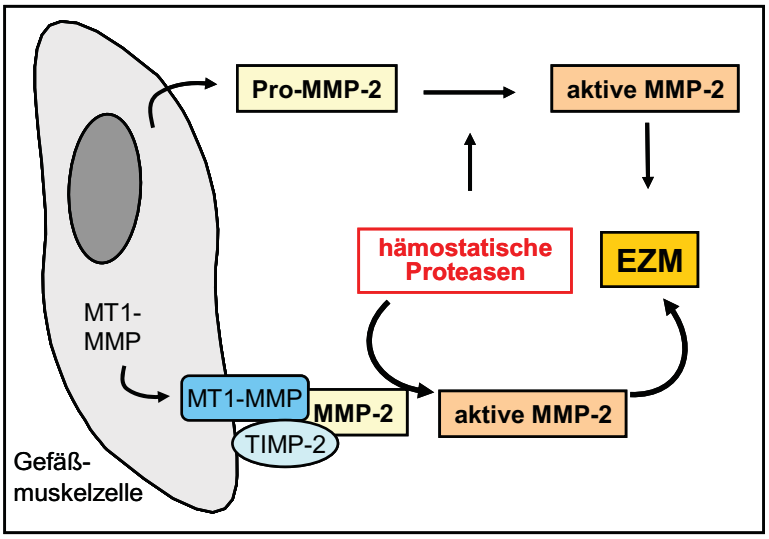

Abb. 5: Aktivierungswege der MMP-2 durch hämostatische Proteasen. Plasmin, Thrombin und FXa können eine Spaltung der Pro-MMP-2 zur aktiven MMP-2 bewirken. Hierdurch können sie zu Umbauvorgängen der extrazellulären Matrix (EZM) betragen. In **Arbeit #1** wurde gezeigt, dass FXa nicht nur eine direkte Spaltung der Pro-MMP2, sondern auch eine Freisetzung von der Zelloberfläche humaner Gefäßmuskelzellen bewirkt (94), wo sie im Komplex mit MT1-MMP und TIMP-2 gebunden vorliegt.

Zur Untersuchung der biologischen Bedeutung der gefundenen FXa-induzierten MMP-2-Aktivierung wurde die FXa-stimulierte DNA-Synthese als Maß für die mitogene Wirkung in humanen Gefäßmuskelzellen und für die Einwanderung in ein Matrigel zur Simulierung der Zellinvasion in An- und Abwesenheit von MMP-Inhibitoren untersucht. Es zeigte sich, dass die FXa-induzierte Mitogenese und die Matrix-Invasion in Anwesenheit eines MMP-Inhibitors gehemmt waren (94). Folglich scheint die FXa-bedingte MMP-Aktivierung an diesen Prozessen, welche zum Krankheitsgeschehen, z.B. der Atherosklerose oder der Invasion von Tumoren, beitragen können, beteiligt zu sein.

In zusätzlichen Untersuchungen wurde geklärt, ob die gefundene MMP-2-Aktivierung durch FXa tatsächlich unabhängig von Thrombin ist. In Abbildung 6A ist dargestellt, dass sowohl FXa, als auch Thrombin eine Spaltung der Pro-MMP-2 und somit ihre Aktivierung bewirken. Untersucht wurde die Aktivierung der MMP-2 mittels Gelzymographie. Dabei werden die gebildeten MMPs in einem gelatine-haltigen Elektrophoresegel aufgetrennt. Die Gelatine wird angefärbt und an den Stellen, an denen sie von der MMP abgebaut wurde, bleiben ungefärbte Bereiche zurück (Abb. 6A). Während der Thrombin-spezifische Inhibitor Hirudin die Thrombin-induzierte MMP-2-Aktivierung verhindert, hat Hirudin keinen Effekt auf die FXa-bedingte Aktivierung der MMP-2. Abbildung 6B stellt den beobachteten Effekt der FXa-induzierten MMP-abhängigen Wanderung von Zellen anhand eines Migrationsmodells dar, welches die Wundheilung simuliert.

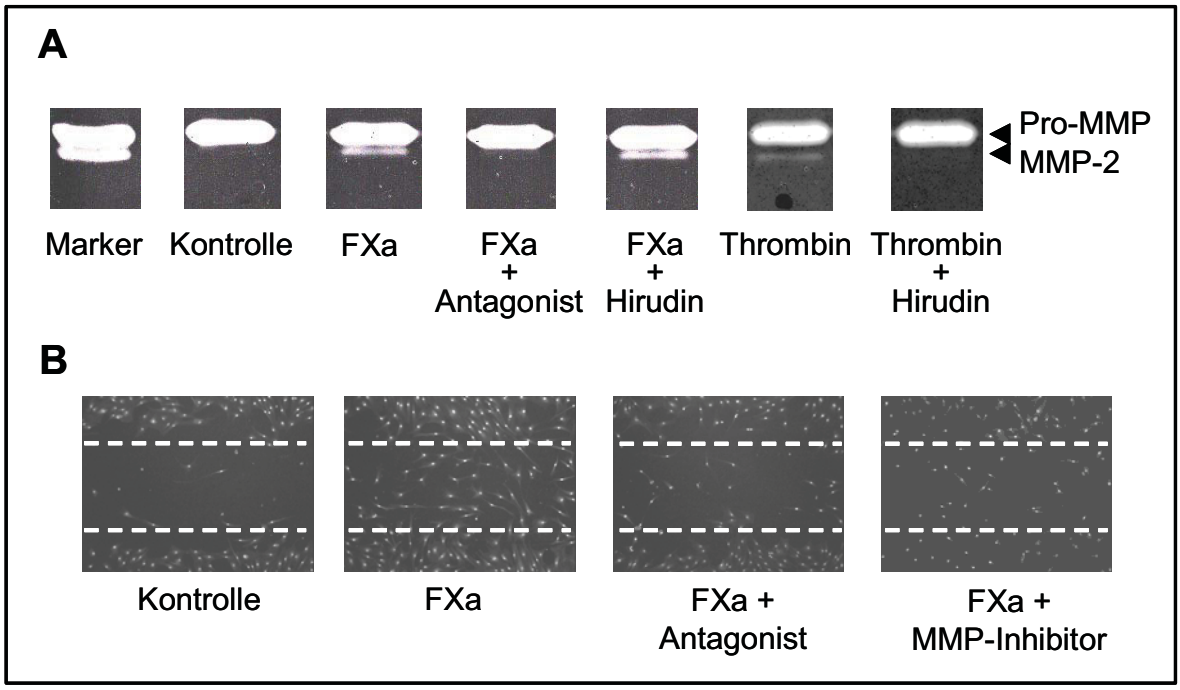

Abb 6: A, Gelatine-Zymographie zur Analyse der MMP-2-Aktivierung. Nach Inkubation mit FXa (100 nM) oder Thrombin (1 U/ml) kommt es zur Aktivierung des Proenzyms, erkennbar an der Ausbildung der kleinern Spaltform. Pro-MMP-2 ist *in vivo* enzymatisch inaktiv und wird im Gel durch Anwesenheit von SDS (Sodiumdodecylsulfat) aktiviert; die kleinere Spaltform ist die aktive MMP-2. Ein spezifischer FXa-Antagonist (DX-9065a, 10 µM) kann die FXa-induzierte Aktivierung der MMP-2 hemmen, der Thrombin-Hemmstoff Hirudin (10 µM) dagegen nicht. **B**, FXa (100 nM) stimuliert die Einwanderung von Gefäßmuskelzellen in ein Verletzungsareal. Durch den FXa-Hemmstoff und einen MMP-Inhibitor (GM6001, 100 nM) wurde dieser Effekt gehemmt (unveröffentlichte Daten).

Schlussfolgerungen

In Arbeit #1 konnte erstmals eine Aktivierung der MMP-2 durch FXa – unabhängig von seiner Thrombin-aktivierenden Wirkung - und die Bedeutung dieses Mechanismus für Mitogenese und Matrixinvasion humaner glatter Gefäßmuskelzellen nachgewiesen werden. Damit kann FXa möglicherweise zur Pathogenese von Gefäß- und Tumorerkrankungen beitragen. Eine Hemmung von FXa könnte über die antikoagulatorische Wirkung hinausgehende positive Effekte bei diesen Erkrankungen zeigen.

2.1.2 Wirkungen von FXa auf die MMP-9

Neben der Wirkung auf die MMP-2 wurde untersucht, ob FXa eine Aktivierung der MMP-9 bewirkt, welche ebenfalls an zahlreichen Erkrankungen, beispielsweise der Plaqueruptur oder Entzündungsprozessen beteiligt ist (89). Es zeigte sich - in

Übereinstimmung mit der Literatur - dass MMP-9 von Gefäßmuskelzellen nur dann vermehrt gebildet wurde, wenn diese mit entzündlichen Stimuli wie Tumor-Nekrose-Faktor-α (TNFα) inkubiert wurden (21). Es fande sich jedoch auch unter diesen proinflamatorischen Bedingungen keine Aktivierung der MMP-9 durch FXa in kultivierten Gefäßmuskelzellen, sondern allenfalls eine Tendenz, die Synthese von MMP-9 in Kombination mit TNFα und anderen Wachstumsfaktoren wie PDGF (platelet-derived growth factor) zu verstärken (hier nicht dargestellt).

2.1.3 Wirkungen von Thrombin auf die Synthese von Hyaluronsäure

Arbeit #2 Van den Boom M, Sarbia M, von Wnuck Lipinski K, Mann P, Meyer-Kirchrath J, Rauch BH, Grabitz K, Levkau B, Schrör K, Fischer JW. Differential regulation of hyaluronic acid synthase isoforms in human saphenous vein smooth muscle cells: possible implications for vein graft stenosis. *Circ Res* 2006; 98:36-44.

Hyaluronsäure (HA) ist ein Polysacharid, bestehend aus sich wiederholenden Disaccharideinheiten (β-1,3-N-Acetylglukosamine-β-1,4-D-Glukuronsäure), welches an der Zellmembran durch drei HA-Synthasen (HAS1-3) gebildet und in den Extrazellularraum abgegeben wird (138). HA ist ein Hauptbestandteil des atherosklerotischen Gewebe, wird im Rahmen der Restenose nach Gefäßverletzung vermehrt gebildet und ist mit proliferierenden Gefäßmuskelzellen und Thrombosen erodierter Plaques assoziiert (59, 103).

In **Arbeit #2** wurden Bedeutung und molekulare Regulation der HA-Synthese in Vena saphena-Bypässen, wie sie in der Herz- und Gefäßchirurgie eingesetzt werden, untersucht. Ein Vergleich von frisch explantierten Venen vor ihrer Implantation als Bypass, mit Gefäßen, die einige Zeit als arterieller Bypass gedient hatten und aus Untersuchungen des Institutes für Pathologie des Universitätsklinikums Düsseldorf stammten, zeigte, dass eine stark vermehrte HA-Bildung in den arterialisierten Gefäßen stattgefunden hatte (141). Da die Faktoren, welche die Induktion der HA-Synthese im Rahmen der Arterialisierung der venösen Bypässe bewirken, nicht bekannt sind, wurden in nachfolgenden *in vitro* Untersuchungen in kultivierten venösen Gefäßmuskelzellen verschiedene Stimuli

eingesetzt, von denen bekannt ist, dass sie die Proliferation von Gefäßmuskelzellen, die EZM Synthese oder Entzündungsprozesse in Gefäßen regulieren (141). Die in dieser Arbeit eingesetzten Faktoren umfassten vasodilatierende Prostaglandine (Iloprost und PGE_2), Thrombin, PDGF, und die Enzündungsmediatoren IL-1β und TGF-β1. PDGF (platelet-derved growth factor, siehe Punkt 2.2.1), ein Wachstumsfaktor, für den eine Induktion der HAS2, der hauptsächlich in Gefäßmuskelzellen exprimierten Isoform der HA-Synthasen, beschrieben ist (33), führte auch in den untersuchten humanen venösen Zellen zu einer vermehrten Bildung von HAS2 sowie zu einer HA-Synthese. Ebenfalls untersucht wurde der Einfluss von Prostaglandinen auf die HAS-Expression. Prostaglandine sind lipidartige Signalmoleküle, die an zahlreichen physiologischen Körperfunktionen, aber auch bei pathologischen Vorgängen, wie der Schmerzvermittlung, an Entzündungsprozessen und der Entwicklung von Gefäßerkrankungen beteiligt sind (74, 118-120). Es fand sich eine deutliche Induktion der HAS1 und der HAS2 durch die beiden Prostaglandine Iloprost und PGE_2. Das deutet auf eine wichtige Funktion dieser Entzündungsmediatoren bei der Anpassung der venösen Gefäße an die höheren arteriellen Druckverhältnisse hin.

Da es in venösen Bypass-Gefäßen häufig zur Aktivierung von Thrombin und zu Thrombosen kommt, wurde auch die Beteiligung von Thrombin für die Regulation der HAS-Enzyme untersucht. Thrombin führte ebenfalls zu einer Induktion der HAS2-Isoform, welche durch den Thrombinrezeptor PAR-1 vermittelt war, hatte jedoch keinen Einfluss auf die HAS1 und die HAS3. (141).

Schlussfolgerungen

Die in venösen Bypässen vermehrt stattfindende Bildung von Thrombin führt nicht nur zur Auslösung von Thrombosen, sondern kann auch zur vermehrten Bildung von HA in der Gefäßwand beitragen. Dies unterstützt die Proliferation glatter Gefäßmuskelzellen und die Anpassung der Bypassgefäße an die erhöhten arteriellen Druckverhältnisse, mag aber auch die häufig auftretende Stenosierung dieser Gefäße fördern.

2.1.4 Effekte von Fibrinogen auf humane glatte Muskelzellen

Arbeit #3 Rauch BH, Müschenborn B, Weber AA, Schrör K.
ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells.
(*Eur J Pharmacol*, 2007; Manuskript im Druck)

Fibrinogen ist ein lang gestrecktes Molekül von 340 kDa Größe. Es besteht aus je zweimal drei Polypeptidketten, den α-, β-, γ-Ketten. Nach proteolytischer Abspaltung der Fibrinopeptide A und B durch Thrombin (128) kommt es zur Polymerisierung und Ausbildung eines Fibringerinnsels (siehe auch Punkt 1.1). Neben seiner Bedeutung für die Blutgerinnung übt Fibrinogen wichtige Effekte auf zelluläre Interaktionen, Entzündungsreaktionen, die Wundheilung und bei der Bildung von Neoplasien aus (77, 128). Darüber hinaus wird Fibrinogen an den Prädilektionsstellen der Atherosklerose in die Gefäßwand eingelagert und eine Hyperfibrinogenämie wird als unabhängiger Risikofaktor für die Entwicklung einer Atherosklerose angesehen (58).

Aus der Literatur ist bekannt, dass Fibrinogen über Bindung an das Adhäsionsmolekül ICAM-1 (intercellular adhesion molecule-1) die Anhaftung von Leukozyten am Endothel und deren transendotheliale Migration in die Gefäßwand fördert (27). ICAM-1 wird ebenfalls von glatten Gefäßmuskelzellen exprimiert. Es wird im Rahmen von Gefäßerkrankungen vermehrt gebildet (12). Da Fibrinogen auch in Gefäßmuskelzellen die Migration (79) anregt und Fibrinogen-Abbauprodukte die Proliferation glatter Muskelzellen stimulieren (80), wurde in **Arbeit #3** die Fragestellung untersucht, ob Fibrinogen seine Wirkungen auf Gefäßmuskelzellen ebenfalls über die Bindung an ICAM-1 ausübt.

Es zeigte sich, dass Fibrinogen eine konzentrations-abhängige Stimulation der Zellmigration humaner glatter Gefäßmuskelzellen bewirkte. Diese konnte durch neutralisierende Antikörper gegen ICAM-1 gehemmt werden (s. Abb. 6 und Arbeit #3). Die verwendeten Fibrinogenkonzentrationen lagen in einem Bereich, wie er auch beim Menschen vorkommt. Das unterstreicht die mögliche Bedeutung für physiologische und in erhöhten Konzentrationen für pathophysiologische Prozesse.

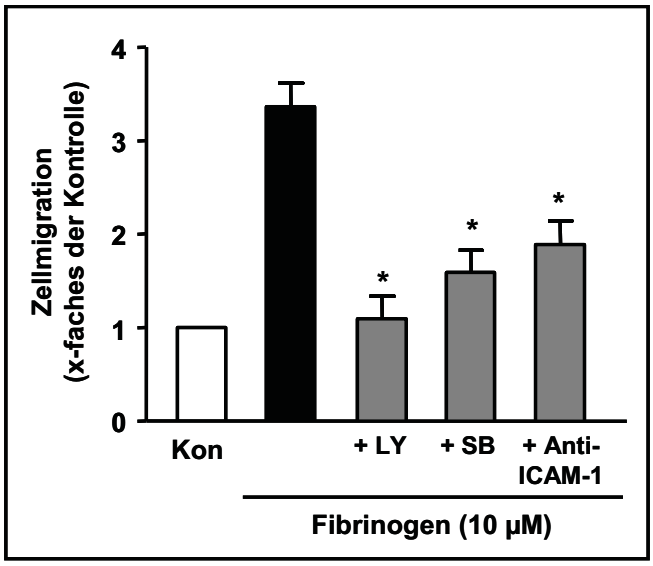

Abb. 7:

Fibrinogen stimuliert die Migration humaner Gefäßmuskelzellen. ICAM-1-neutralisierende Antikörper (Anti-ICAM-1, 10 µg/ml) vermindern diesen Effekt, ebenso wie Hemmstoffe der Proteinkinase B (Akt) (LY, 10 µM) und der p38 MAPK (SB, 10 µM). Diese deutet auf eine Beteiligung dieser intrazellulären Signalwege an der Fibrinogen-induzierten Migration der Muskelzellen hin; n=8, *p<0,05 versus unbehandelte Kontrolle (Kon) (modifiziert nach Arbeit #3).

In Arbeit #3 wurden auch die intrazellulären Signalwege untersucht, die an der Fibrinogen-induzierten Migration von Gefäßmuskelzellen beteiligt sind. Hierbei ließ sich eine Beteiligung der Proteinkinase B (auch als Akt bezeichnet), sowie der p38-MAPK nachweisen. Diese beiden Moleküle besitzen zentrale Funktionen intrazellulärer Signalwege, die typischerweise an Proliferation und Migration von Zellen beteiligt sind und häufig gemeinsam aktiviert werden (9). Im Gegensatz zur ausgeprägten Stimulation der Zellmigration durch Fibrinogen, war dessen Wirkung als mitogener Stimulus in humanen Gefäßmuskelzellen eher gering (s. Arbeit #3).

Schlussfolgerungen

Fibrinogen besitzt nicht nur eine Bedeutung im Rahmen der Blutstillung als Substrat des Thrombins, sondern wirkt auch direkt auf Gefäßmuskelzellen. Die Fibrinogen-stimulierte Wanderung von Gefäßmuskelzellen hängt von der Bindung an ICAM-1 und der nachfolgenden Aktivierung des Akt- und p38-MAPK-Signalweges ab. Über diesen Mechanismus kann Fibrinogen zur Entwicklung von Gefäßerkrankungen beitragen.

2.2 Freisetzung von FGF-2 durch Thrombin und FXa

Die wachstumsfördernde Wirkung von Thrombin auf glatte Gefäßmuskelzellen ist seit längerem belegt (34). In Arbeiten aus unserem Labor wurde zudem die mitogene Wirkung von FXa nachgewiesen (13, 15). Zusätzlich wird durch beide Faktoren eine verstärkte Migration glatter Gefäßmuskelzellen ausgelöst (94, 96). Über diese Wirkungen können Thrombin und FXa zur Pathogenese von Gefäßerkrankungen beitragen (s. Punkt 1.2.) (127). 1999 wurde erstmals beschrieben, dass die Aktivierung eines sekundären Rezeptors, des epidermalen Wachstumsfaktor-Rezeptors (EGFR, epidermal growth factor rezeptor), an den mitogenen Effekten von Thrombin beteiligt ist (90). Nach Aktivierung der G-Protein-gekoppelten PARs durch Thrombin kommt es unter Beteiligung einer Metalloproteinase zur Freisetzung des so genannten heparin-binding EGF-like factor (HB-EGF), welcher an den EGFR bindet und diesen aktiviert (54, 55). Der EGFR ist eine Rezeptortyrosinkinase (RTK), welche nach Bindung eines extrazellulären Liganden - hier des HB-EGF - seine intrazelluläre Kinase-Domäne aktiviert und die Phosphorylierung weiterer intrazellulärer Signalmoleküle bewirkt. In Arbeiten aus dem Labor von Alexander Clowes (University of Washington, Seattle) wurde erstmals gezeigt, dass die Aktivierung dieses Signalweges an der Thrombin-induzierten Migration in Gefäßmuskelzellen der Ratte beteiligt ist (54, 55). In eigenen Arbeiten des Autors wurde die mögliche Bedeutung dieses Mechanismus für die Thrombin-induzierte Mitogenese in humanen Gefäßmuskelzellen untersucht. Jedoch ließ sich ein solcher EGF-abhängiger Mechanismus in humanen Zellen nicht belegen. Stattdessen wurde die Beteiligung eines anderen RTK-Systems - Freisetzung von Fibroblastenwachstumsfaktor-2 (FGF-2) und die Aktivierung des FGF-Rezeptors - für die mitogene Wirkung von Thrombin und auch FXa in humanen Gefäßmuskelzellen gefunden (Punkt 2.2.1).

2.2.1 Thrombin- und FXa-induzierte Mitogenese und Migration in humanen glatten Muskelzellen

Arbeit #4 Rauch BH, Millette E, Kenagy RD, Daum G, Clowes AW. Thrombin- and factor-Xa-induced DNA synthesis is mediated by transactivation of fibroblast growth factor receptor-1 in human vascular smooth muscle cells. *Circ Res* 2004; 94:340-345.

Der basische Fibroblastenwachstumsfaktor (bFGF oder FGF-2) ist ein intrazellulär gespeichertes Molekül. Es kann aus Zellen freigesetzt werden und bindet an Heparin bzw. das endogene Pendant, die Heparan-Sulfat-Proteglykane (HSPGs) in der EZM (85, 151). Die Bedeutung von FGF-2 als Wachstumsfaktor für Gefäßmuskelzellen und für die Entwicklung der Atherosklerose und Restenose, aber auch für Angiogenese und die Emryonalentwicklung, sind in zahlreichen Studien *in vitro* und *in vivo* belegt (51, 68, 85, 99, 100). FGF-2 gehört zu einer Familie von 23 bekannten Fibroblastenwachstumsfaktoren, welche an vier verschiedene RTKs, die FGF-Rezeptoren (FGFR-1 bis -4), binden (85, 102). In Gefäßmuskelzellen stellt der FGFR-1 den Hauptvertreter dieser Rezeptoren dar (85, 102). Interessant ist, dass trotz des Fehlens der erforderlichen Amonisäuresequenz für die Externalisierung über den Golgi-Apparat, FGF-2 aus Zellen freisetzt werden kann (82). Kürzlich wurde von eine anderen Arbeitsgruppe auch die Thrombin-indzierte Freisetzung des weiteren Mitgliedes der FGF-Familie, dem FGF-1, beschrieben (26). Diese Beobachtung unterstreicht die Bedeutung einer Thrombin-regulierten Freisetzung sekundärer Wachstumsfaktoren für die Gefäßbiologie und möglicher weiterer Zellfunktionen als generellen Mechanismus, über den Thrombin und womöglich auch FXa pathophysiologische Vorgänge beeinflussen kann.

In **Arbeit #4** wurde die Freisetzung von FGF-2 aus Gefäßmuskelzellen der menschlichen Aorta durch Thrombin und FXa erstmals beschrieben. Es konnte gezeigt werden, dass beide Faktoren eine rasche Freisetzung von FGF-2 in die extra- bzw. perizelluläre Matrix bewirken. Dort bindet das FGF-2 an die Heparin-artigen Seitenketten der HSPGs auf der Zelloberfläche (s. auch Arbeit #7). Diese Bindung ist für eine effektive Aktivierung des spezifischen FGF-Rezeptors - in Gefäßmuskelzellen vornehmlich der FGFR-1 - erforderlich und bewirkt dessen

Autophosphorylierung. In Abbildung 8 ist die Thrombin-induzierte Freisetzung von FGF-2 und die Phosphorylierung des FGFR-1 nach Stimulation der humanen Gefäßmuskelzellen mit Thrombin exemplarisch dargestellt. Für FXa wurden vergleichbare Ergebnisse gefunden (Arbeit #4).

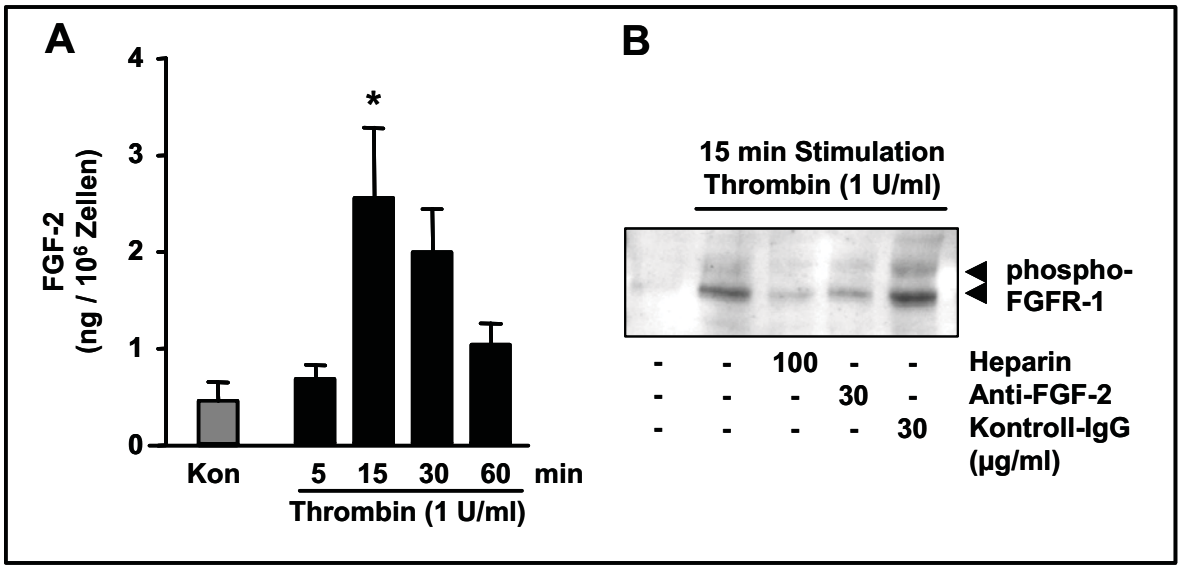

Abb. 8: Freisetzung von FGF-2 und autokrine Phosphorylierung des FGF-Rezeptor-1 durch Thrombin in humanen aortalen Gefäßmuskelzellen. **(A)** Thrombin bewirkte eine Freisetzung von FGF-2 nach extrazellulär innerhalb von 15 min nach Stimulation der Zellen mit Thrombin; n=4, *p<0,05 vs. Kontrolle (Kon). **(B)** Nach Inkubation mit Thrombin für 15 min wurden die Zellen lysiert und der FGFR-1 mittels spezifischer Antikörper immunopräzipitiert. Die Phosphoylierung wurde mittels phospho-spezifischer Anti-Tyrosin-Antikörper im Western Blot bestimmt (Pfeile). Vergleichbare Ergebnisse wurden für FXa gefunden (modifiziert nach Arbeit #4).

Die Aktivierung des FGF-Signalweges ist für die Thrombin- und FXa-induzierte Mitogenese erforderlich. Exogen zugesetztes Heparin oder FGF-2-neutralisierende Antikörper können das freigesetzte FGF-2 binden und die Aktivierung der FGFR verhindern. Hierdurch wird die mitogene Wirkung von Thrombin und FXa gehemmt (Arbeit #4).

2.2.2 Rolle der Protease-aktivierten Rezeptoren

Die Freisetzung von intrazellulärem FGF-2 durch Thrombin und FXa wird über PARs vermittelt. In Arbeit #4 und auch in Arbeit #7 konnte dies belegt werden. Hierzu wurden spezifische PAR-aktivierende Peptide (PAR-APs) eingesetzt. Diese entsprechen den Sequenzen der endogenen „angebundenen“ Liganden, die nach proteolytischer Spaltung der extrazellulären Rezeptorendigungen freigelegt

werden, und bewirken eine Autoaktivierung der Rezeptoren (42, 70, 86). In Arbeit #4 führte die Inkubation humaner aortaler Gefäßmuskelzellen mit PAR-1-AP zu einer Stimulation der DNA-Synthese, die durch FGF-2-neutralisierende Antikörper und durch Heparin gehemmt wurde. In Arbeit #4, wurde das freigesetzte FGF-2 direkt bestimmt und eine Freisetzung innerhalb von 10 min nach Stimulation mit PAR-APs gefunden. Abbildung 9 stellt die beschriebenen Vorgänge der Thrombin und FXa-induzierten FGF-2 Freisetzung und die nachfolgende FGFR-Aktivierung schematisch dar.

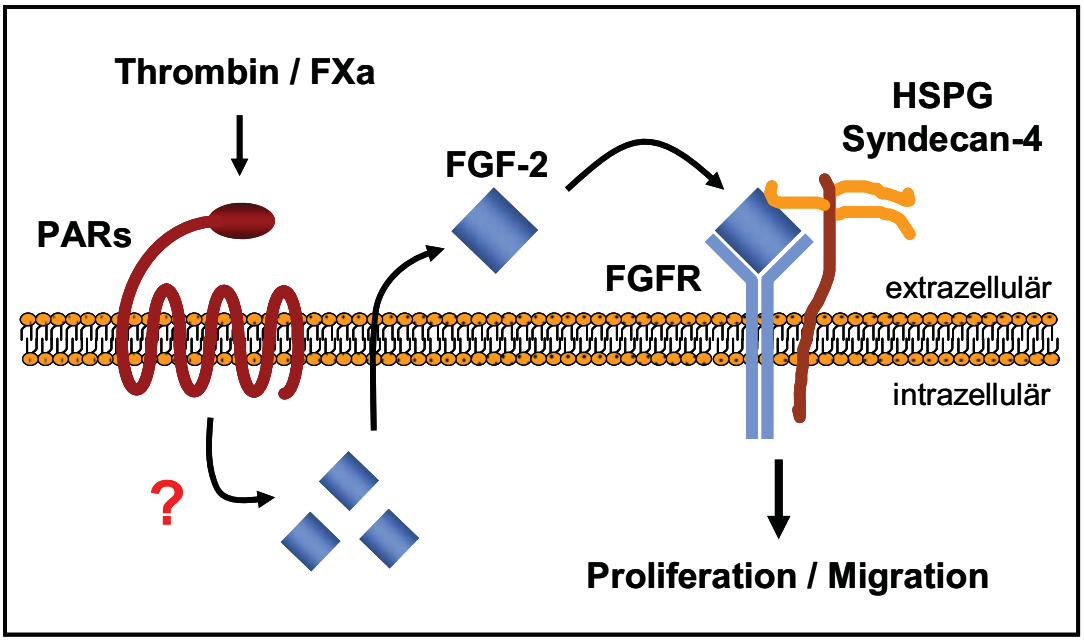

Abb 9: Thrombin und FXa induzieren über die Protease-aktivierten Rezeptoren (PARs) eine Freisetzung von intrazellulär gespeichertem FGF-2 in die EZM mit nachfolgender Aktivierung des FGF-Rezeptor-1 (FGFR) (95). Hierfür ist Syndecan-4, ein HSPG (Heparan-Sulfat-Proteoglykan) erforderlich. Es bindet das freigesetzte FGF-2 und funktioniert als Kofaktor für den FGFR (96).

Schlussfolgerungen

Es konnte ein neuer Wirkmechanismus aufgeklärt werden: Thrombin und FXa bewirken über eine Aktivierung der PARs eine rasche Freisetzung von FGF-2 nach extrazellulär. Die nachfolgende Aktivierung des FGFR-1 trägt zur mitogenen Wirkung von Thrombin und FXa in humanen Gefäßmuskelzellen bei.

2.2.3 Wirkung von Heparin und Bedeutung von Syndecan-4

Arbeit #5 Millette E, Rauch BH, Kenagy RD, Daum G, Clowes AW. Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. *Circ Res* 2005; 96: 172-179.

Arbeit #6 Rauch BH, Millette E, Kenagy RD, Daum G, Fischer JW, Clowes AW. Syndecan-4 is required for thrombin-induced migration and proliferation in human vascular smooth muscle cells. *J Biol Chem* 2005; 280:17507-17511.

In **Arbeit #5** wurde untersucht, ob auch andere Wachstumsfaktoren, welche die Proliferation von Gefäßmuskelzellen stimulieren, eine Freisetzung von FGF-2 und eine Aktivierung des FGFR-1 bewirken. PDGF (platelet-derived growth factor) wird aus Blutplättchen freigesetzt oder auch von Zellen der Gefäßwand gebildet und ist an der Entstehung der Atherosklerose und Restenose beteiligt (76, 99). In Arbeit #5 haben wir die Wirkung einer bestimmten Isoform, PDGF-BB (105), in humanen Gefäßmuskelzellen untersucht. Die durch PDGF-BB stimulierte Zellproliferation der Muskelzellen wurde durch Heparin und durch FGF-2-neutralisierende Antikörper gehemmt. Das lässt auf die Beteiligung eine FGF-2-Freisetzung auch für die Wirkungen von PDGF-BB schließen. Allerdings gab es große Unterschiede im Vergleich zur Thrombin- und FXa-induzierten FGF-2 Freisetzung. Während Thrombin und FXa eine rasche Freisetzung von FGF-2 innerhalb von 10 - 15 Minuten und ebenso eine rasche Phosphorylierung von FGFR-1 bewirken (s. Arbeiten #4 und #7), führte PDGF-BB erst nach 2 Stunden zu einer messbaren FGF-2-Freisetzung (Arbeit #5). Dies deutet auf unterschiedliche Freisetzungs-mechanismen für FGF-2 hin, die durch die jeweiligen Stimuli ausgelöst werden. In Arbeit #5 wurde die Beteiligung des PI3-Kinase-Akt-Weges, einem PDGF-abhängig regulierten pro-entzündlichen Signalweg (98), und der Proteinkinase C dokumentiert. Im Gegensatz dazu wird die FGF-2-Freisetzung durch Thrombin und FXa über andere Signalwege reguliert. Deren Untersuchung ist Gegenstand der derzeitigen Forschung in unserem Labor (s. Punkt 2.2.4). Beiden Freisetzungswegen, den durch Thrombin und FXa bzw. den durch PDGF induzierten, ist jedoch die Hemmung des FGF-2 Signals durch Heparin gemein. Dieser Aspekt wurde in Arbeit #6 genauer untersucht.

In Abbildung 9 ist dargestellt, dass für die Aktivierung des FGFR die Bindung von FGF-2 an HSPGs erforderlich ist, welche die Funktion von Kofaktoren besitzen. In **Arbeit #6** wurde die mögliche Beteiligung eines bestimmten HSPG, des Syndecan-4, untersucht. Syndecan-4 ist deshalb von besonderem Interesse, weil es als Kofaktor für den in Arbeit #4 untersuchten FGFR-1 beschrieben ist und einzigartige Eigenschaften zur Regulation intrazellulärer Signalwege besitzt (Abb. 10). Syndecan-4 besteht aus einem Kernprotein. Dieses ist in der Zellmembran verankert. Es besitzt einen extrazellulären Anteil, der Heparan-Sulfat-Seitenketten trägt, und einen intrazellulären Teil, über den Syndecan-4 nach Bindung von FGF-2 und Interaktion mit dem FGFR-1 intrazelluläre Signalwege, wie den Proteinkinase C (PKC)-Weg, aktivieren und darüber mitogene Wirkungen vermitteln kann (93, 137).

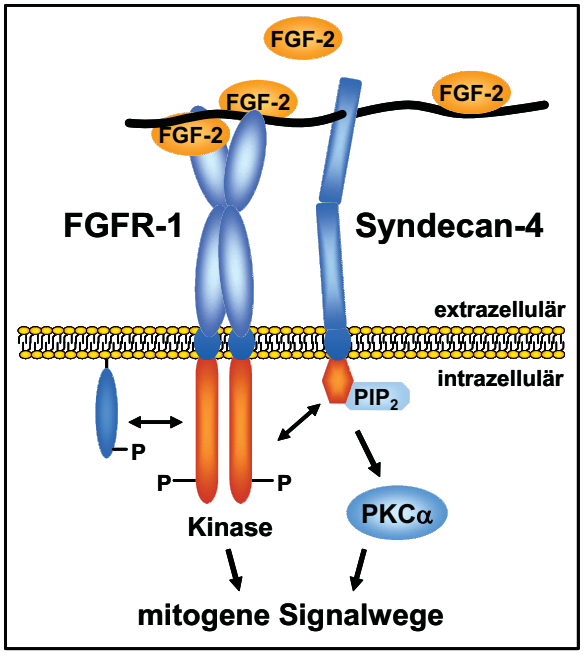

Abb. 10:

FGF-2 bindet an die extrazellulären Heparan-Sulphat (HS)-Seitenketten des Syndecan-4. Dies führt zur Assoziation von Syndecan-4 mit dem FGFR-1. Nachfolgend kommt es zur Phophorylierung (P-) des intrazellulären Kinaseanteils des FGFR-1 und zur Bindung einer an der Membraninnenseite liegenden Phosphatase, die ihrerseits den intrazellulären Anteil des Syndecan-4 dephosphoryliert. Dies ermöglicht die Bindung des „second messanger" Moleküls Phosphatidylinositol 4,5-Bisphosphate (PIP_2), wodurch eine direkte Bindung und Aktivierung der Proteinkinase C-alpha (PKCα) erfolgen kann. PKCα kann dann weitere, z.B. mitogene Signalwege, induzieren (modifiziert nach Rapraeger, 2000 (93) und Simons, 2001 (125)).

Um die mögliche Bedeutung des Syndecan-4 für die Thrombin-induzierten Mitogenese und Migration in humanen Gefäßmuskelzellen zu untersuchen, wurde in Arbeit #6 eine relativ neue Technologie angewandt. Um die Expression von Syndecan-4 zu hemmen und somit dessen Funktion beurteilen zu können, wurde „short interfering RNA" (siRNA), auch RNA-Interferenz genannt, eingesetzt (30, 31, 75, 96). Dabei werden kurze doppelsträngige RNA-Sequenzen in Zellen eingebracht. Durch Induktion eines speziellen Proteinkomplexes bewirkt dies den

Abbau der endogenen RNA, welche der siRNA-Sequenz entspricht. Praktisch führt dies dazu, dass gezielt Gene in Zellen „abgeschaltet“ werden und so ihre Rolle für die Zellfunktion untersucht werden kann. Mittels dieser Technik konnte in Arbeit #6 die Expression von Syndecan-4 erstmals in humanen Gefäßmuskelzellen untersucht und seine Bedeutung für die Thrombin-induzierte Proliferation und Migration gezeigt werden (Abb. 11).

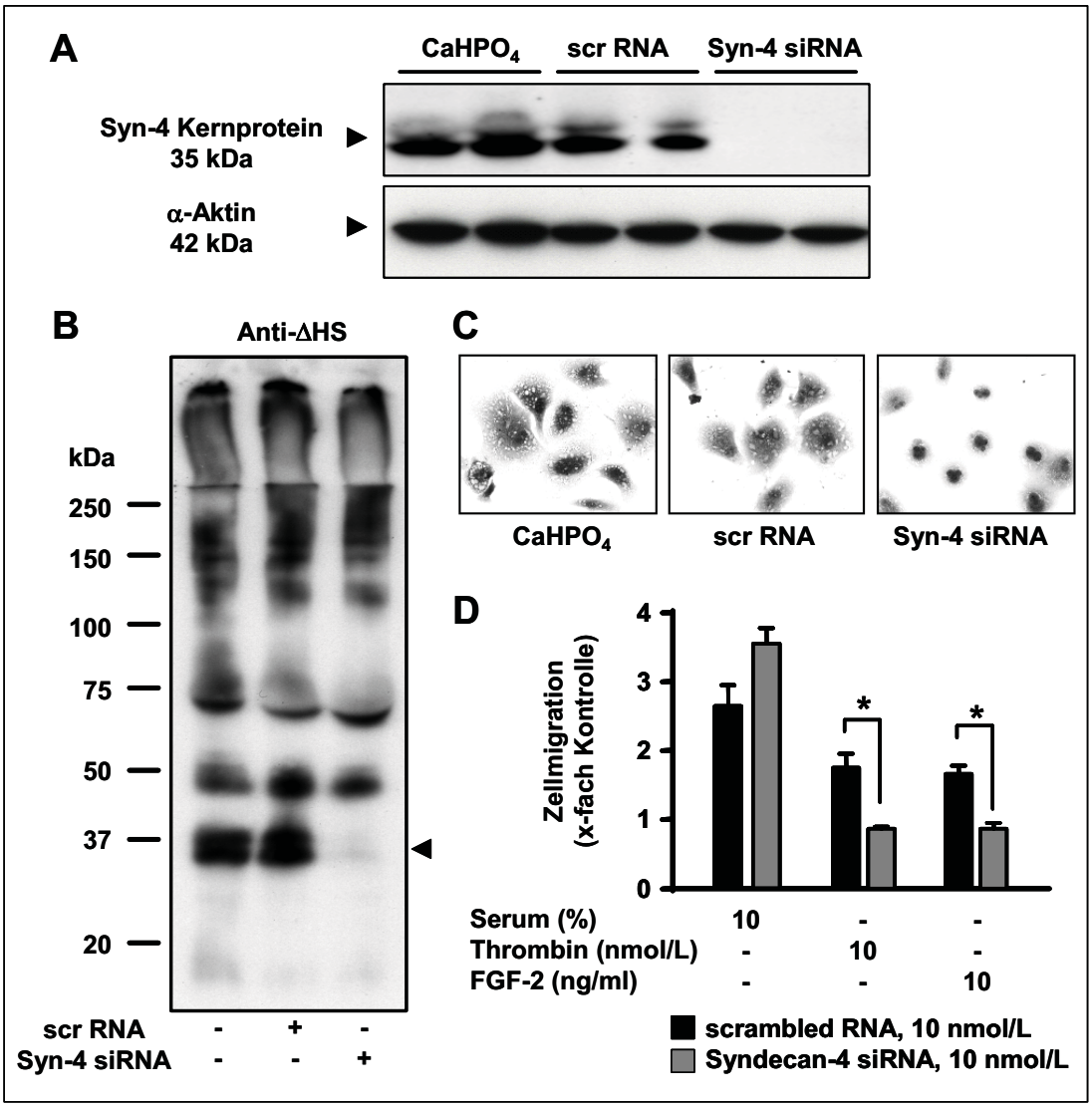

Abb. 11: Effekte spezifischer siRNA auf die Expression von Syndecan-4 und den Phänotyp humaner Gefäßmuskelzellen. **A:** Darstellung des Syndecan-4 (Syn-4) Kernproteins mittels Western Blot nach Abdauung der HS-Seitenketten und seine Expressionshemmung durch siRNA im Vergleich mit Transfektionsvehikel allein (Kalziumphosphat, $CaHPO_4$) und unspezifischer Kontroll-RNA (scrambled (scr) RNA). Zum Vergleich der aufgetragenen Proteinmengen wurde α-Aktin bestimmt. **B:** Um mögliche unspezifische Effekte der siRNA auf andere HSPGs auszuschließen, wurden ein Antikörper (Anti-ΔHS) verwendet, der abgedaute HS-Stümpfe an Kernproteinen erkennt. Es zeigte sich kein unspezifischer Effekt der Syn-4-siRNA, da nur das Syn-4-Protein gehemmt wurde (Pfeil). **C:** Der Mangel von Syn-4 verlangsamte die Ausbreitung der Zellen nach dem Aussähen in Kulturschalen, hier dargestellt nach 1 h. **D:** Syn-4-siRNA hemmte die Thrombin- und die FGF-2-induzierte Migration in humanen Gefäßmuskelzellen, nicht jedoch die durch Serum stimulierte (modifiziert nach Arbeit #6) (96).

Wie in Abbildung 11 gezeigt, konnte mittels siRNA die Expression des Syndecan-4 in humanen Gefäßmuskelzellen reduziert werde. Hierdurch wurde die Fähigkeit dieser so behandelten Zellen, auf Thrombin als Stimulus mit zu reagieren, gehemmt. Davon waren sowohl die Thrombin-induzierte Migration (Abb. 11), als auch die mitogene Wirkung des Thrombin betroffen (Arbeit #6). Diese Ergebnisse belegten, dass die Anwesenheit von Syndean-4 für die zellulären Wirkungen von Thrombin in humanen Gefäßmuskelzellen erforderlich ist.

Schlussfolgerungen

Die hier dargestellten Untersuchungen konnten erstmals die Bedeutung des Proteoglykan Syndecan-4 für die Effekte von Thrombin in humanen Gefäßmuskelzellen und somit auch seine mögliche Bedeutung für die Entwicklung von Gefäßerkrankungen beim Menschen zeigen. Effiziente pharmakologische Maßnahmen, die mit FGF-2 Wirkungen in der Gefäßwand interferieren, könnten einen therapeutischen Nutzen bei der Behandlung von Gefäßerkrankungen wie der Restenose oder der Progression der Atherosklerose erbringen.

2.2.4 Mitogene Wirkung von Thrombin in Cholesterin-beladenen glatten Gefäßmuskelzellen

Arbeit #7 Rauch BH, Scholz GA, Baumgärtel-Allekotte D, Censarek P, Fischer JW, Weber AA, Schrör K.
Cholesterol Enhances Thrombin-induced Release of Fibroblast Growth Factor-2 in Human Vascular Smooth Muscle Cells. *Arterioscler Thromb Vasc Biol* 2007; 27:e20-e25.

In weiteren Untersuchungen sollte eine mögliche Bedeutung des Cholesterol-spiegels für den beschriebenen Mechanismus der Thrombin-induzierten FGF-2-Freisetzung untersucht werden. Ein Grund für die Entwicklung der Hypothese, dass es einen Zusammenhang zwischen zellulären Thrombinwirkungen, FGF-Freisetzung, Cholesterol und mitogener Wirkung geben könnte, war, dass in der Literatur ein Zusammenhang von Cholesterol und FGF-Signalwegen beschrieben

ist. Außerdem ist die Bedeutung erhöhter Low-Density-Lipoprotein (LDL)-Werte, dem Transportweg des Cholesterins in die Organe, seit langem als Risikofaktor für die Entwicklung der Atherosklerose bekannt. Darüber hinaus zeigen klinische Untersuchungen, dass es einen Zusammenhang zwischen erhöhten Cholesterolspiegeln und vermehrter Restenoserate nach Gefäßverletzung, wie der Ballondehnung und nach Bypasschirurgie, gibt (18, 22, 101). Experimentelle Studien belegen zudem, dass Cholesterol die mitogenen Wirkungen von Wachstumsfaktoren wie PDGF verstärken kann (112), und in Gefäßmuskelzellen der Ratte wurde eine vermehrte Bildung von FGF-2 durch eine Cholesterol-Erhöhung beschrieben (60). Ob Cholesterol jedoch die Thrombin-induzierten Wirkungen in humanen Gefäßmuskelzellen beeinflusst, war bislang nicht bekannt. Daher wurde in **Arbeit #7** ein Modell entwickelt, mit dem Effekte erhöhter intrazellulärer Cholesterolspiegel in humanen Gefäßmuskelzellen untersucht werden konnten. Die Zellen wurden mit einem Komplex aus Methyl-β-Cyclodextrin inkubiert. Es nimmt Cholesterol in seinem hydrophoben Inneren auf, bewirkt eine Lösungsvermittlung des ansonsten in wässrigen Lösungen unlöslichen Cholesterol und führt zur Anreicherung von Cholesterol in den Zellen (106). Die Wirkung dieser Methode auf die Gefäßmuskelzellen ist in Abbildung 12 dargestellt.

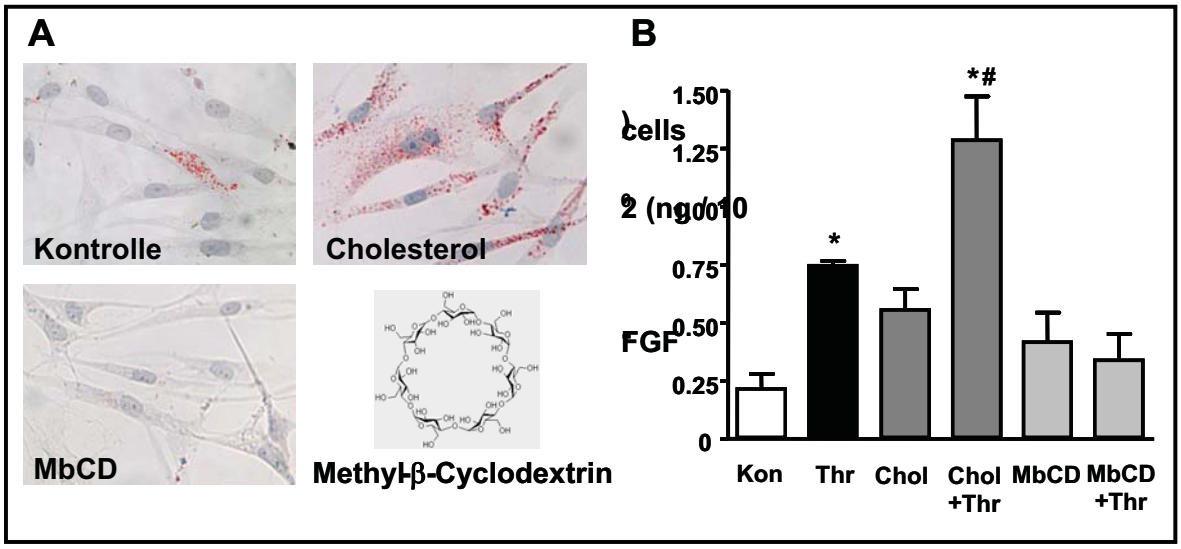

Abb. 12: Humane glatte Gefäßmuskelzellen wurden mittels Methy-β-Cyclodextrin (MbCD), einem ringförmigen Zuckermolekül, mit Cholesterol angereichert. **A:** Nach Inkubation mit Cholesterol-MbCD (10 µg/ml) oder MbCD in entsprechender Menge für 24 h, erfolgte die Anfärbung des intrazellulären Cholesterol mit Öl-Rot. **B:** Die Beladung der Zellen mit Cholesterol bewirkte eine vermehrte FGF-2-Freisetzung, die durch Stimulation mit Thrombin (Thr, 3 U/ml, 10 min) stark gesteigert wurde. MbCD ohne Zusatz von Cholesterol hingegen hemmte die Thrombin-induzierte Freisetzung von FGF-2; n=4, *p<0.05 vs. Kontrolle (Kon), #p<0.05 vs. Thr ohne Cholesterol. Modifiziert nach Arbeit #7 (97).

In den Untersuchungen der Arbeit #7 zeigte sich, dass die Beladung der humanen Gefäßmuskelzellen mit Cholesterol (Abb.12A) zu einer vermehrten Freisetzung von FGF-2 nach extrazellulär führte (Abb. 12B). Als Ursache bzw. zugrunde liegender Mechanismus dieser vermehrten FGF-2-Freisetzung wurde eine durch Cholesterol verursachte Erhöhung der Produktion von FGF-2 ermittelt. Cholesterol induzierte die FGF-2 mRNA- und Protein-Synthese in humanen Gefäßmuskelzellen (Arbeit #7). Dieser Effekt des Cholesterols beeinflusste auch die Reaktion der Zellen auf die Stimulation mit Thrombin. Die Thrombin-induzierte DNA-Synthese und Zellproliferation waren in den mit Cholesterol angereicherten Zellen deutlich erhöht. Als ursächlich hierfür ließ sich die vermehrte FGF-2-Synthese durch das erhöhte zelluläre Cholesterol belegen (97).

2.2.5 Bedeutung des Rho-GTPase Signalweges

In aktuellen Studien wird untersucht, wie Thrombin und FXa FGF-2 aus humanen Gefäßmuskelzellen freisetzen. Dazu wurden zahlreiche Inhibitoren von Signalwegen eingesetzt, die an der Proliferation von Zellen beteiligt sind oder für die eine Bedeutung für die Freisetzung von Proteinen von intra- nach extrazellulär beschrieben wurde (s. auch Arbeit #7). Von verschiedenen eingesetzten Substanzen, konnten nur zwei eine Reduktion der Thrombin- und FXa-induzierten FGF-2 Freisetzung erreichen – der Rho-Kinase Inhibitor Y27632 und der Proteinkinase C-delta (PKCδ)-Inhibitor Rottlerin. Andere Substanzen, z.B. unspezifische PKC-Inhibitoren, ein Tyrosinkinase-Inhibitor oder Stoffe, die mit dem Zytoskelett interferieren, oder Hemmstoffe des Kalziumstoffwechsels zeigten keinen Effekt auf die Freisetzung von FGF-2 (unveröffentlichte Daten). Hieraus kann geschlossen werden, dass der Rho-Signalweg, es handelt sich um kleine GTP-bindende Signalmoleküle, für die eine Beteiligung an der Thrombin-stimulierten Zellproliferation beschrieben wurde, an der FGF-2-Freisetzung beteiligt ist. Die Rho-GTPasen umfassen im Wesentlichen drei Moleküle, Rho A, Rac1 und CDC42. Sie sind an zahlreichen Zellfunktionen beteiligt sind (113, 123, 144, 150). Nachgeschaltete, durch diese Moleküle aktivierte Kinasen sind die Rho-Kinase (ROK) sowie auch die PKCδ, welche durch Rho A bzw. ROK aktiviert wird (65). Die kleinen Rho-GTPasen können durch verschiedene Wachstumsfaktoren

und auch PAR-abhängig durch Thrombin aktiviert werden (s. auch Abb.4, Punkt 1.3) (113). Sie sind an der Regulation verschiedener Prozesse wie Entzündungen, dem Gefäßwachstum und auch an der Externalisierung von intrazellulären Proteinen beteiligt (3, 32). Während die Bedeutung von Rho A für zelluläre Wirkungen des Thrombin schon länger beschrieben ist (113, 123), gibt es bislang keine Untersuchungen darüber, ob FXa ebenfalls Rho A aktivieren kann. In Abbildung 12 ist die Aktivierung von Rho A durch FXa mittels eines spezifischen Rho A-Aktivierungsassays dargestellt. Dabei wird der Effekt ausgenutzt, dass aktiviertes Rho A an ein bestimmtes Bindeprotein, das Rhotekin, bindet (145). Über eine Markierung mit dem Protein GST, welches wiederum an ein Trägergel bindet, kann der Anteil des nach Stimulation der Zellen mit FXa aktivierten Rho A aus dem Lysat der Zellen isoliert werden („pull down"-Assay). Die Bestimmung des durch FXa aktivierten Rho A erfolgte dann mittels Western Blot (Abb. 13).

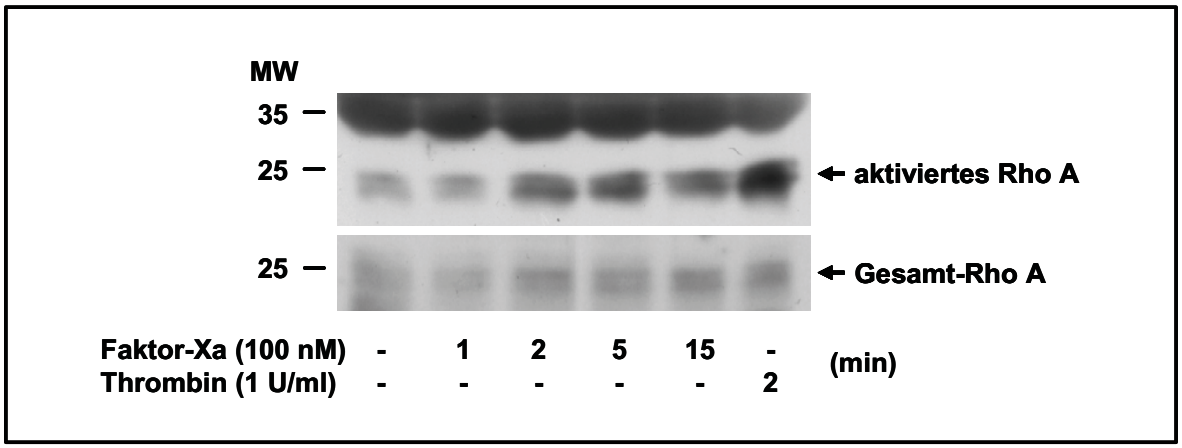

Abb. 13: FXa aktiviert Rho A in humanen Gefäßmuskelzellen. Humane glatte Gefäßmuskelzellen wurden mit FXa (100 nM) oder mit Thrombin (1 U/ml) inkubiert. Der Anteil des aktivierten Rho A wurde mittels „pull down"-Assay, bei die Bindung des aktiven Rho A an seinen Bindepartner Rhotekin ausgenutzt wird, bestimmt (unveröffentlichte Daten).

Die in Arbeit #7 beschriebene vermehrte Thrombin-induzierte FGF-Freisetzung in Cholesterol-reichen Gefäßmuskelzellen wurde durch Hemmstoffe von Rho A und der durch Rho A aktivierten ROK gehemmt, was auf die Beteiligung dieses Signalweges an der Freisetzung von FGF-2 schließen lässt. Die Bedeutung dieses Signalweges für die FXa-induzierte Freisetzung von FGF-2 aus humanen Gefäßmuskelzellen ist derzeit Gegenstand weiterer Untersuchungen.

Schlussfolgerungen

Es konnte erstmals gezeigt werden, dass die mitogene Wirkung von Thrombin in humanen glatten Gefäßmuskelzellen vom intrazellulären Cholesterolgehalt der Zellen abhängt und bei erhöhten Spiegeln verstärkt ist. Cholesterol scheint die Zellproliferation indirekt über die Synthese und Freisetzung von FGF-2 zu regulieren. In weiteren mechanistischen Untersuchungen wurde gezeigt, dass der Rho/Rho-Kinase Signalweg an der FGF-2-Freisetzung beteiligt ist. Über diese Mechanismen könnten erhöhte Cholesterolspiegel beim Patienten zur Entwicklung von Gefäßerkrankungen beitragen. Ob eine mögliche zukünftige pharmakologische Interaktion mit dem Rho-Kinase Signalweg die Therapie von Patienten mit Gefäßerkrankungen verbessern kann, bleibt abzuwarten.

2.3 Regulation des Protease-aktivierten Rezeptor-1 (PAR-1)

Arbeit #8 Pape R, Rauch BH, Rosenkranz AC, Kaber G, Schrör K.
Prostacyclin inhibits expression of protease-activated receptor-1 PKA-dependently in human vascular smooth muscle cells.
(Manuskript in Revision bei *Arterioscler Thromb Vasc Biol*.)

Der klassische Thrombinrezeptor PAR-1 ist ubiquitär exprimiert. Neben seiner Bedeutung für die Aktivierung von Thrombozyten (s. auch Punkt 1.3) besitzt er wichtige Funktionen während der Embryonalentwicklung (25, 71). Er wird von fast allen Körperzellen gebildet und spielt - neben seinen zahlreichen physiologischen Funktionen - eine wichtige Rolle bei der Entwicklung von Erkrankungen. PAR-1 wird in atherosklerotisch veränderten Gefäßen (6, 127), aber auch von Tumorgeweben vermehrt gebildet (70, 86). Weitere Bedeutung besitzt er für die Angiogenese und für die Regulation der Endothelintegrität, indem er die Durchlässigkeit (Permeabilität) des Endothels für Bestandteile des Blutes reguliert (19, 35, 57). Hierüber kann Thrombin bzw. PAR-1 beispielsweise die Durchlässigkeit der Blut-Hirn-Schranke beeinflussen (84). Während die Funktionen des PAR-1 intensiv untersucht wurden, sind die Regulationsmechanismen, die seine Expression in den verschiedenen Geweben steuern, kaum erforscht. In **Arbeit #8** wird die Regulation des PAR-1 durch vasodilatierende Prostaglandine beschrieben. Prostaglandine sind Schmerz- und Entzündungsmediatoren, die von Gefäßzellen gebildet werden können (74, 118-120). Im Rahmen von Entzündungen kommt es zur vermehrten Bildung dieser Mediatoren, da das Schlüsselenzym ihrer Synthese, die Cyclooxygenase-2 (74), vermehrt gebildet wird und die Produktion von Prostaglandinen somit gesteigert ist. Durch COX-2-abhängig gebildete Prostaglandine kann auch die Expression von Proteinen, die an der Wirkung von Gerinnungsfaktoren beteiligt sind, beeinflusst werden. Kürzlich wurde in unserer Arbeitsgruppe die Regulation von Thrombomodulin, einem wichtigen endogenen Inhibitor der Blutgerinnung, durch COX-2-abhängig gebildete Prostaglandine in Gefäßmuskelzellen beschrieben (91). Die Arbeit #8 untersucht die Bedeutung der Prostglandine Prostazyklin und PGE_1 für die transkriptionelle Regulation des PAR-1.

2.3.1 Transkriptionale Regulation durch Prostazyclin

Prostazyklin und PGE_1 stellen die vorwiegend von Gefäßmuskelzellen gebildeten Prostaglandine dar. Im Rahmen von Entzündungen, die mit einer Induktion der COX-2 einhergehen, werden sie vermehrt gebildet (44, 91). In **Arbeit #8** wird beschrieben, dass insbesondere das Prostazyklin-Analogon Iloprost (10) eine Hemmung der Expression des PAR-1 bewirkt. Es konnte eine starke Hemmung der mRNA-Bildung, eine Verminderung der insgesamt in der Zelle vorhandenen PAR-1 Proteinmenge und eine verminderte Expression von PAR-1 an der Zelloberfläche nachgewiesen werden (Abb. 14). Während Iloprost in Konzentrationen wirksam war, die physiologischen Bereichen entsprechen, mussten im Gegensatz dazu relativ hohe PGE_1-Konzentrationen eingesetzt werden, um vergleichbare Effekte zu erzielen, was auf eine Bedeutung vornehmlich von Prostazyklin für die Regulation des PAR-1 hindeutet.

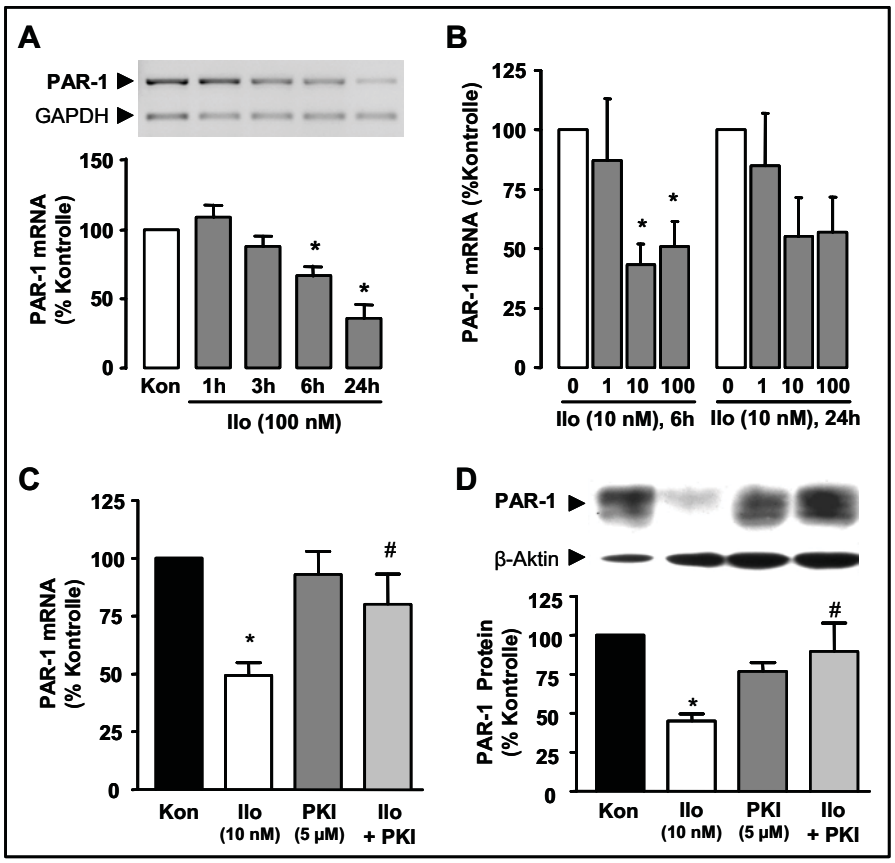

Abb 14: Regulation der PAR-1-Expression durch das Prostazyklin-Analogon Iloprost. Iloprost (Ilo) bewirkte eine Reduktion der PAR-1 mRNA im Zeitverlauf über 24 h in humanen Gefäßmuskelzellen. Dieser Effekt wurde **(A)** mittels semiquantitativer PCR (Ilo 100 nM) und **(B)** mittels quantivativer real-time PCR (Ilo 10 nM) nachgewiesen. Diese Hemmung der Expression von PAR-1 mRNA **(C)** und Protein **(D)** konnte durch den spezifischen Proteinkinase A Hemmstoff PKI verhindert werden, wenn er den Zellen 30 Minuten vor Inkubation mit Ilo zugesetzt wurde; n=4-7 Versuche, *p<0,05 vs. Kontrolle (Kon), ,p<0,05 vs. Ilo (modifiziert nach Arbeit #8).

Die hemmende Wirkung von Iloprost auf die PAR-1-Expression wurde erst im Verlauf mehrerer Stunden nach Inkubation der untersuchten humanen Gefäßmuskelzellen mit Iloprost beobachtet (Abb.14). Dies deutet darauf hin, dass die Reduktion der PAR-1 Expression durch Iloprost eher auf eine Verminderung von Mechanismen, welche die PAR-1-Expression erhöhen, als auf einen aktiven Abbau des PAR-1 in den Zellen zurückzuführen ist. Die an dieser Regulation beteiligten Mechanismen sind Gegenstand aktueller Untersuchungen.

2.3.2 Rolle der Proteinkinase A

Es zeigte sich, dass der second messanger cAMP (cyclisches Adenosin-Monophosphat) an der Regulation des PAR-1 beteiligt sind. Substanzen die wie Iloprost (37) eine Erhöhung des intrazellulären cAMP bewirken, verursachten ebenfalls eine Reduktion der PAR-1-Expression. In weiteren Untersuchungen wurde daher die Beteiligung der Proteinkinase A (PKA), die durch cAMP aktiviert wird (36), an der Regulation des PAR-1 untersucht. Es zeigte sich, dass eine spezifische Hemmung der PKA (87) die Iloprost-vermittelte Reduktion der PAR-1 Expression aufheben konnte. Das belegt die Beteiligung der PKA an der Regulation des PAR-1 belegt (Abb. 14). In zusätzlichen Studien wurde die Expression des PAR-1 auf der Zelloberfläche mittels Durchflusszytometrie untersucht. Auch hierbei fand sich ebenfalls eine Hemmung der PAR-1-Expression in Abhängigkeit von der PKA (hier nicht dargestellt, s. Arbeit #8).

2.3.3 Funktionelle Bedeutung der Regulation des PAR-1

Um die Bedeutung der Prostaglandin-regulierten PAR-1 Expression in humanen Gefäßmuskelzellen zu untersuchen, wurden die Zellen mit Iloprost entweder für 24 Stunden oder nur für 1 Stunde inkubiert. Dann wurde die mitogene Wirkung von Thrombin und des spezifischen PAR-1-aktivierenden Peptides (PAR-1-AP) in vorbehandelten und nicht vorbehandelten Zellen untersucht. Diese erfolgte durch Bestimmung der DNA-Syntheserate anhand des Einbaus von radioaktiv markiertem Thymidin ([^{3}H]-Thymidin, Abbildung 15).

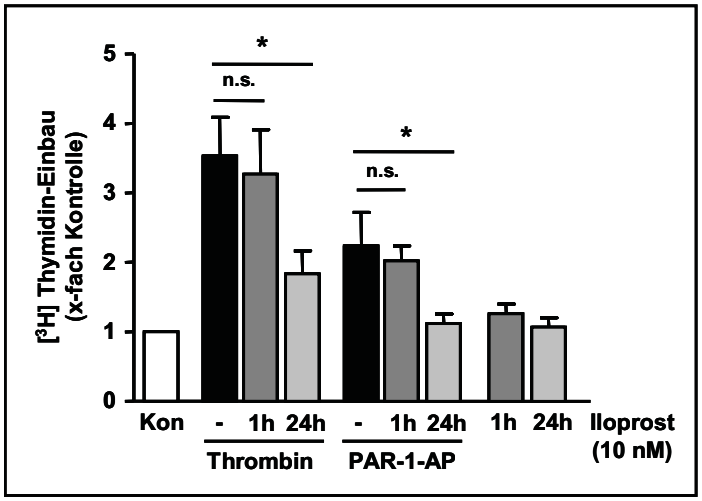

Abb. 15:

Die Effekte von Iloprost auf die durch Thrombin (3 U/ml) und durch das PAR-1-aktivierende Peptid (PAR-1-AP, 100 µM) ausgeübte Mitogenese wurde mittels Einbaus von [^{3}H]-Thymidin ermittelt. Während eine nur kurzzeitige Vorbehandlung (1 h) mit Iloprost (Ilo, 10 nM) keine Wirkung auf die Mitogenese zeigte, führte die längerfristige Vorbehandlung der Zellen (24 h) zu einer Reduktion der mitogenen Wirkung von Thrombin und PAR-1-AP (modifiziert nach Arbeit #8).

Aus der beobachteten Hemmung der Thrombin- und PAR-1-AP-stimulierten Mitogenese kann geschlossen werde, dass die Reduktion der PAR-1-Expression durch Prostaglandine eine protektive Wirkung vor den proliferativen Wirkungen des Thrombin bewirkt.

Schlussfolgerungen

Vasodilatierende Prostaglandine - vornehmlich Prostazyclin - hemmen über einen cAMP-PKA-abhängigen Mechanismus die zelluläre Expression des Thrombinrezeptor PAR-1. Über diesen Regelkreis können sie die mitogene Wirkung von Thrombin in humanen Gefäßmuskelzellen modulieren. Die mögliche Bedeutung dieser Regulierung des PAR-1, der an der Entwicklung von Gefäßerkrankungen und Entzündungsvorgängen beteiligt ist, ist in weiteren Studien zu klären. Ob die Hemmung der Expression des PAR-1 durch Prostagandine einen möglichen therapeutischen Nutzen erbringen kann, ist ebenfalls in zukünftigen Untersuchungen zu hinterfragen.

3 Perspektiven

3.1 Entwicklung neuer Antikoagulantien

Die Entwicklung neuer gerinnungshemmender Medikamente stellt ein wichtiges Ziel zur Verbesserung der antikoagulatorischen Therapie dar (116). Verschiedene neue Gerinnungshemmer sind derzeit in experimenteller und klinischer Entwicklung. Die vordringliche Aufgabe besteht darin, Substanzen zu finden, die einen ausreichenden Schutz vor Thrombosen und ihren Komplikationen bieten, bei möglichst geringem Risiko schwerer Blutungen. Dass eine Hemmung der Blutgerinnung immer mit einem erhöhten Blutungsrisiko einhergeht, versteht sich einerseits von selbst, andererseits sind viele Aspekte der sehr komplexen Blutgerinnung erst unzureichend verstanden. Es besteht daher noch ein erheblicher Forschungsbedarf, um die Abläufe der Gerinnung unter pathologischen Bedingungen besser zu verstehen und darauf aufbauend mögliche neue Prinzipien zur Thromboseprophylaxe beim Patienten entwickeln zu können (117).

Die beiden Schlüsselenzyme der Gerinnung, Thrombin und FXa, welche für die Ausbildung eines Fibringerinnsels unabdingbar sind, stehen dabei im Mittelpunkt des Bestrebens, neue Antikoagulanzien zu entwickeln (38, 53). Ein Beispiel eines neuen und oral anwendbaren Thrombin-Inhibitors war das Melagatran. Diese Substanz wurde zunächst als mindestens ebenso sicher wie die niedermolekularen Heparine eingeschätzt und als erste mögliche orale alternative Therapie angesehen (64). Allerdings musste die Substanz im Februar 2006 wieder vom Markt zurückgezogen werden, da sich eine erhöhte Lebertoxizität zeigte (136). Es bleibt zu hoffen, dass weitere neue Thrombin-Hemmstoffe, die dringend benötigt werden und z.T. bereits in klinischer Erprobung sind (7), bessere klinische Eigenschaften besitzen (136). Eine weitere viel versprechende Substanz ist ein neuer FXa-Inhibitor, das Rivaroxaban (53, 62, 139). Bisherige Ergebnisse lassen auf eine gute Anwendbarkeit als Antikoagulanz hoffen, jedoch bleibt auch hier die endgültige Beurteilung der Eigenschaften dieses oral verfügbaren kompetetiven FXa-Inhibitors bezüglich seiner potentiellen Nebenwirkungen erst noch abzuwarten.

3.2 Wirkungen von Antikoagulantien auf Zellen der Gefäßwand

Die in dieser Arbeit dargelegten Untersuchungen der Wirkungen von Gerinnungsfaktoren auf die Zellen der Gefäßwand, legen nahe, dass neue Antikoagulanzien nicht nur in die Blutstillung eingreifen, sondern auch zahlreiche Wirkungen auf die Gefäßwand ausüben können (48). Die neuen niedermolekularen direkten Thrombin- und FXa-Antagonisten lassen eine bessere Wirksamkeit auf ein bereits gebildetes Blutgerinnsel bzw. auf die Effekte eines Klots auf die Gefäßwand erwarten (siehe auch Punkt 1.5) (48). Diese könnte einerseits zu einer besseren Wirksamkeit diese Substanzen auf Veränderungen des Gefäßes beitragen. Andererseits könnten diese Substanzen aber auch mit einem veränderten Spektrum an unerwünschten Wirkungen aufwarten, als es von den bislang angewendeten Stoffen, z.B. den Heparinen, bekannt ist. Die Erforschung weiterer zellulärer Wirkungen von Gerinnungsfaktoren ist daher sinnvoll und wird auch weiterhin einen Schwerpunkt in unserer Arbeitsgruppe darstellen. Neben den Effekten auf Gefäßmuskelzellen, kann Thrombin auch Einflüsse auf das Endothel ausüben. So kann Thrombin die Durchlässigkeit der Endothelzellschicht steigern (Abb. 16) und hierdurch zum Übertritt von Bestandteilen des Blutes in das Gewebe beitragen. Die Ausbildung von Ödemen kann dadurch begünstigt werden.

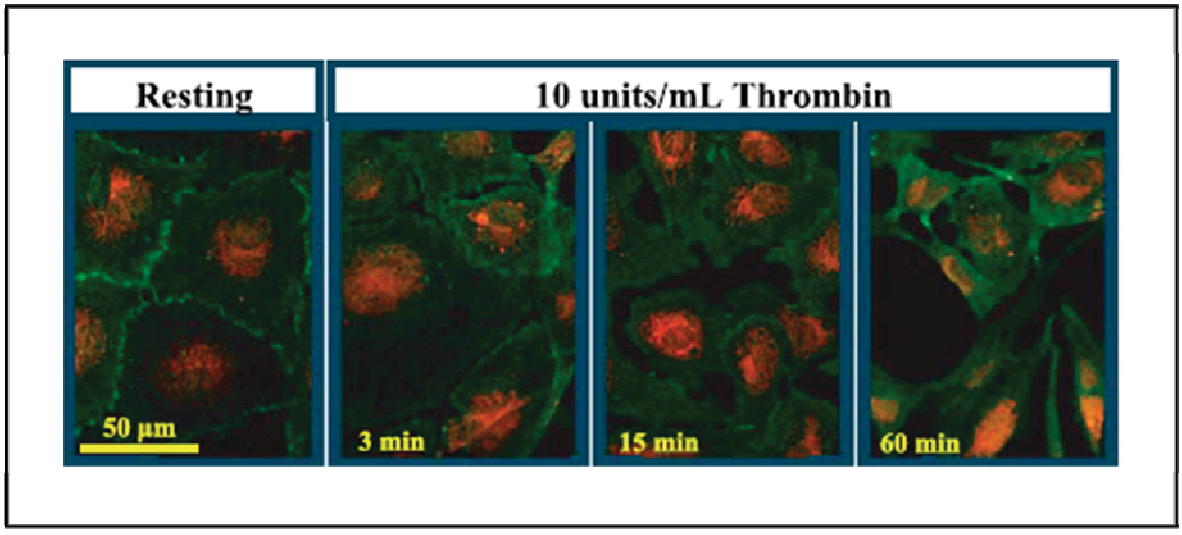

Abb. 16: Effekte von Thrombin auf das Endothel. Diese aus der Arbeit von K. Nobe et al., 2006 stammende Abbildung stellt „Fenster“ in einer Endothezellschicht dar, die sich nach Inkubation der Zellen mit Thrombin innerhalb der angegebenen Zeiten öffnen (84). Hierdurch fördert Thrombin die Ausbildung von Ödemen nach einer Verletzung. Ob Hemmstoffe des Thrombin, die derzeit als neuartige Antikoagulantien entwickelt werden, auch diese Effekte des Thrombin hemmen und hierdurch beispielsweise eine Ödembildung vermindern können, muss abgewartet werden.

Die Wirkungen von Gerinnungsfaktoren auf die Gefäßwand stellen wichtige Reparaturmechanismen dar, über die Schäden am Gefäßsystem behoben werden sollen. Die weitere Untersuchung der Bedeutung von Thrombin und FXa beispielsweise für die Integrität des Endothels oder eventuelle weitere Wirkungen an glatten Gefäßmuskelzellen sind daher sinnvoll und notwendig, um Wirkungen neuer Hemmstoffe dieser Faktoren einschätzen und verbesserte Therapien entwickeln zu können. Darüber hinaus bieten direkte Inhibitoren von Thrombin und FXa mögliche alternative Anwendungsgebiete, die zunächst nicht mit Antikoagulantien in Verbindung gebracht werden.

3.3 Alternative Einsatzgebiete von Gerinnungshemmern

Neben der Entstehung von Thrombosen ist eine Bedeutung von Thrombin und FXa sowie ihren zellulären Rezeptoren, den PARs, belegt (70, 86). In verschiedenen Studien wurden Effekte von Thrombin- und FXa-Inhibitoren auf die Tumorentwicklung und Metastasierung gezeigt (78). Einen Hinweis darauf, dass zelluläre Wirkungen von FXa unabhängig von ihrer koagulatorischen Wirkung relevant sind, gibt die Arbeit von Banke et al. (5), in welcher ein FXa-Hemmstoff die Tumormetastasierung in Konzentrationen hemmt, in denen nur eine geringe antikoagulatorische Wirkung bestand. Darüber hinaus ist bekannt, dass Heparine auch unabhängig von ihrer antikoagulatorischen Funktion hemmende Eigenschaften auf Tumorwachstum und Metastasenbildung besitzen (61). Diese Wirkungen sind auf direkte zelluläre Effekte, wie die Hemmung der Wirkung von Wachstumsfaktoren zurückzuführen (61). Die in dieser Arbeit beschriebene Thrombin- und FXa-induzierte Freisetzung von FGF-2, einem potenten Wachstumsfaktor auch für Tumorzellen (28), kann hierbei relevant sein. Eine ebenfalls wichtige Funktion für neoplastische Erkrankungen kommt der hier beschriebenen Aktivierung von Matrixmetalloproteinasen (MMPs) zu (142). Eine Hemmung der MMP-Aktivierung durch Thrombin- und FXa-Antagonisten könnte eine hemmende Wirkung auf Tumorwachstum und Metastasenbildung erbringen.

Weitere Bedeutung könnte zukünftigen Antagonisten von Thrombin und FXa als Hemmstoffen der Gefäßneubildung (Angiogenese) zukommen. Die Neubildung von Gefäßen besitzt für das Wachstum von Tumoren eine wichtige Funktion, da ohne diesen Vorgang eine Ausbildung und Versorgung solider Tumore nicht stattfinden kann. Die Bedeutung von Gerinnungsfaktoren für die Angiogenese und für die Entwicklung von Tumorerkrankungen ist in verschiedenen Untersuchungen belegt (83, 111).

Ein weiteres Gebiet, in dem sich die zukünftige Anwendung von Medikamenten, die in das Gerinnungssystem eingreifen, als therapeutisch wertvoll erweisen könnte, stellen Entzündungen dar. Blutgerinnung und Immunsystem besitzen eine enge Verknüpfung (132). So werde die PARs, die durch Thrombin und FXa aktiviert werden, auf zahlreichen Zellen des Immunsystems exprimiert und sind an der Regulation entzündlicher Reaktionen beteiligt (88, 109). Neue Substanzen, welche die Funktionen der PARs beeinflussen können, wären daher als mögliche Pharmaka zur Behandlung von Entzündungen oder Immunerkrankungen geeignet (88).

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6 Anlagen (Arbeiten #1 - #8)

Arbeit #1 Rauch BH, Bretschneider E, Braun M, Schrör K.
Factor Xa releases matrix metalloproteinase-2 (MMP-2) from human vascular smooth muscle cells and stimulates the conversion of pro–MMP-2 to MMP-2: Role of MMP-2 in Factor Xa–induced DNA synthesis and matrix invasion. *Circ Res* 2002; 90:1122-1127.

Arbeit #2 Van den Boom M, Sarbia M, von Wnuck Lipinski K, Mann P, Meyer-Kirchrath J, Rauch BH, Grabitz K, Levkau B, Schrör K, Fischer JW.
Differential regulation of hyaluronic acid synthase isoforms in human saphenous vein smooth muscle cells: possible implications for vein graft stenosis. *Circ Res* 2006; 98:36-44.

Arbeit #3 Rauch BH, Müschenborn B, Weber AA, Schrör K.
ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells.
(*Eur J Pharmacol*, 2007; im Druck)

Arbeit #4 Rauch BH, Millette E, Kenagy RD, Daum G, Clowes AW.
Thrombin- and factor-Xa-induced DNA synthesis is mediated by transactivation of fibroblast growth factor receptor-1 in human vascular smooth muscle cells. *Circ Res* 2004; 94:340-345.

Arbeit #5 Millette E, Rauch BH, Kenagy RD, Daum G, Clowes AW.
Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. *Circ Res* 2005; 96: 172-179.

Arbeit #6 Rauch BH, Millette E, Kenagy RD, Daum G, Fischer JW, Clowes AW.
Syndecan-4 is required for thrombin-induced migration and proliferation in human vascular smooth muscle cells. *J Biol Chem* 2005; 280:17507-17511.

Arbeit #7 Rauch BH, Scholz GA, Baumgärtel-Allekotte D, Censarek P, Fischer JW, Weber AA, Schrör K.
Cholesterol Enhances Thrombin-induced Release of Fibroblast Growth Factor-2 in Human Vascular Smooth Muscle Cells. *Arterioscler Thromb Vasc Biol* 2007; 27:e20-e25.

Arbeit #8 *Pape R, *Rauch BH, Rosenkranz AC, Kaber G, Schrör K.
Prostacyclin inhibits expression of protease-activated receptor-1 PKA-dependently in human vascular smooth muscle cells.
(Manuscript in Revision bei *Arterioscler Thromb Vasc Biol.*)
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Arbeit #1

Rauch BH, Bretschneider E, Braun M, Schrör K.

Factor Xa releases matrix metalloproteinase-2 (MMP-2) from human vascular smooth muscle cells and stimulates the conversion of pro–MMP-2 to MMP-2: Role of MMP-2 in Factor Xa–induced DNA synthesis and matrix invasion.

Circ Res 2002; 90:1122-1127.

Factor Xa Releases Matrix Metalloproteinase-2 (MMP-2) From Human Vascular Smooth Muscle Cells and Stimulates the Conversion of Pro–MMP-2 to MMP-2

Role of MMP-2 in Factor Xa–Induced DNA Synthesis and Matrix Invasion

Bernhard H. Rauch, Ellen Bretschneider, Marina Braun, Karsten Schrör

Abstract—Pro–matrix metalloproteinase-2 (pro–MMP-2) is expressed in vascular smooth muscle cells (SMCs). We report that activated coagulation factor X (FXa) induces the release of MMP-2 (65 kDa) from human SMCs. In addition, FXa cleaves pro–MMP-2 (72 kDa) into MMP-2. Pro–MMP-2 and MMP-2 were determined by gelatin zymography. MMP-2 was generated in conditioned medium containing pro–MMP-2 in a concentration-dependent fashion by FXa (3 to 100 nmol/L). FX at concentrations up to 300 nmol/L was ineffective. The conversion of pro–MMP-2 to MMP-2 was inhibited by a selective FXa inhibitor (DX-9065a) at 3 to 10 μmol/L. There was a concentration-dependent induction of an intermediate MMP-2 form (68 kDa) in lysates of FXa-treated cells. This indicates that cellular mechanisms are involved in FXa-induced conversion of pro–MMP-2. As a possible biological consequence of MMP-2 activation by FXa, DNA synthesis and matrix invasion of SMCs were determined. Both were stimulated by FXa and inhibited by the selective FXa inhibitor DX-9065a and the MMP inhibitor GM 6001 but not by hirudin or aprotinin. It is concluded that stimulation of SMCs by FXa increases the levels of MMP-2 in the extracellular space and that two different mechanisms are involved: release of active MMP-2 and cleavage of secreted pro–MMP-2. Both might contribute to the mitogenic potency of FXa and FXa-stimulated matrix invasion of SMCs. **(*Circ Res*. 2002;90:1122-1127.)**

Key Words: matrix metalloproteinase-2 ■ factor Xa ■ vascular smooth muscle cells ■ extracellular matrix invasion ■ mitogenesis

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-endopeptidases. MMPs are thought to play an important role in the physiological turnover of extracellular matrix (ECM) components. This includes embryonic tissue morphogenesis, tissue repair, and angiogenesis. In pathological conditions such as atherosclerosis, arthritis, glomerulonephritis, gastric ulcer, tumor invasion, and metastasis, MMPs are also involved in ECM degradation.[1–5] MMPs are synthesized intracellularly and secreted into the extracellular space as proenzymes. The propeptide domain keeps the proenzyme inactive by covalent binding of the catalytic zinc ion. Cell surface–associated urokinase-type plasminogen activator (uPA)/plasmin complex and other MMPs can activate it after proteolytic cleavage.[1,2,6] MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs).[3] Recently, it has been found that membrane-type MMPs (MT-MMPs) can cause MMP activation, leading to the hypothesis of a predominantly pericellular MMP activation cascade.[6]

Recent research has also shown that proliferation and migration of smooth muscle cells (SMCs) are linked to coagulation and fibrinolysis.[7,8] Generation of plasmin causes MMP activation and subsequent ECM breakdown.[9] This is considered as a prerequisite for cell migration into damaged tissues, for example, tumor invasion and tissue remodeling.[4] It has been demonstrated that MMP-2 contributes to cell proliferation, migration, and matrix invasion in a number of cell types such as tumor cells, fibroblasts, and SMCs.[10–13]

In addition to the activation of MMPs by plasmin, activation of MMP-2 by thrombin is also well established.[14,15] However, little is known about the effects of other coagulation factors, such as factor Xa (FXa), on the activation of MMPs. Both, thrombin and FXa are not only key enzymes in blood coagulation but also mitogens in vascular SMCs.[16] Because MMP-2 and MMP-9 are dominant MMPs in the vascular tissue,[5] we have investigated the effects of FXa on these enzymes in vascular SMCs.

Known activation mechanisms for MMP-2 are the cleavage of pro–MMP-2 by MT1-MMP[2,17] or thrombin.[18] Others have demonstrated the cleavage of MMP-2 in the presence of the coagulation factors II, Va, VIIa, and Xa in human umbilical vein endothelial cells.[19] MMP-2 can be induced by

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platelet-derived growth factor (PDGF) in rat SMCs.[12] In addition, it has been shown that MMP-9 can be induced by inflammatory cytokines, such as interleukin (IL)-1α and tumor necrosis factor (TNF)-α in rabbit and human fibroblasts. These effects were enhanced by simultaneous stimulation with PDGF-BB.[20]

We report in the present study that FXa releases MMP-2 from cultured human SMCs. Furthermore, we demonstrate that FXa converts pro–MMP-2 into active MMP-2 in conditioned, cell-free medium. This elevation of extracellular MMP-2 levels by FXa might contribute to its mitogenic potency as well as matrix invasion of SMCs.

Materials and Methods

Materials

Gelatin (porcine skin, 300 bloom), trypsin, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), Triton X-100 was obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM), antibiotics, and fetal calf serum were obtained from Life Technologies (Karlsruhe, Germany). MMP-2 zymography standard, MMP inhibitor GM 6001, and monoclonal anti–MMP-2-antibodies (clone 42-5D11) were purchased from Calbiochem. Coomassie Brilliant Blue R-250 was obtained from Bio-Rad. Human activated and inactive coagulation factors (FXa and FX) were from Kordia (Leiden, The Netherlands). Factor Xa inhibitor DX-9065a was kindly provided by Dr S. Kunitada (Daiichi Pharmaceutical Co, Ltd, Tokyo, Japan). α-Thrombin was kindly provided by Dr J. Stürzebecher (Zentrum für Vaskuläre Biologie und Medizin, Friedrich-Schiller-Universität Jena, Germany). Cell culture materials were purchased from Becton Dickinson.

Cell Culture

Vascular SMCs were isolated from human saphenous veins or human mammary arteries by the explant technique and cultured as previously described.[21] SMCs from passages 4 to 9 were serum-deprived for 72 hours and then stimulated with FX or FXa.

Zymography

SMCs seeded in 24-well plates were harvested with serum-free medium for 72 hours. Media were collected and centrifuged for 10 minutes at 14 000*g* at room temperature to remove detached cells and debris. These conditioned media from unstimulated cells were used to study the actions of FXa and FX in a cell-free system. Alternatively, cultured cells were stimulated with these compounds, and the medium was collected afterward for zymography.

Zymography was performed using 7% SDS/polyacrylamide gels (SDS-PAGE), containing 0.7 mg/mL gelatin. Samples of cell culture medium were resolved in nonreducing Laemmli-buffer (final concentrations: 2% wt/vol SDS, 10% glycerol, 0.0625 mol/L sodium dihydrogen phosphate/disodium hydrogen phosphate, pH 7.0, and 0.01% bromphenol blue). To obtain cell lysates, after stimulation of the cells, they were washed 3 times with phosphate-buffered saline (PBS) and lysed in Laemmli-buffer. Samples were separated by electrophoresis. Then, gels were washed 3 times for 10 minutes at room temperature (50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 2.5% Triton X-100, 0.02% NaN_3) to remove SDS from the gels. Using a modified buffer (1% Triton X-100 instead of 2.5%) gels were incubated for 18 to 36 hours at 37°C. To visualize lytic bands, gels were stained with Coomassie Brilliant Blue R-250 (0.2%) in 40% methanol and 10% acetic acid. Intensity of pro–MMP-2 and MMP-2 bands was quantified using Gel Doc 1000 and software Quantity One, version 4.1.1 (Bio-Rad). After background subtraction, intensity of MMP-2 bands was related to the respective pro–MMP-2 band. This quotient of pro–MMP-2 and MMP-2 signal from unstimulated controls was set to 100% and stimulated cells were referred to control.

Western Blotting

SMCs were seeded in 6-well plates and serum-deprived for 72 hours. Cells were stimulated with FX, FXa, or thrombin for further 24 hours. Media were centrifuged for 10 minutes at 14 000*g* and then lyophilized (freeze dryer Beta I, Christ GmbH) to concentrate MMPs. Electrophoresis (7% SDS-PAGE), blotting of proteins onto polyvinylidene difluoride membranes (Immobilon-P, Millipore), and blocking of membranes in Blotto (Tris-buffered saline, 0.1% Tween-20, 5% wt/vol nonfat dry milk) was carried out as previously described.[21] Membranes were probed with monoclonal MMP-2 antibodies (1:100 in Blotto) and incubated with peroxidase-conjugated secondary antibodies (1:3,000 in Blotto). Bands were visualized by enhanced chemiluminescence (Amersham-Pharmacia Biotech) and quantified by the Gel Doc 1000 system. Quantification was performed in the same way as described above for zymography.

[^{3}H]Thymidine Incorporation

Subconfluent cells were treated with serum-free medium for 24 hours. Cells were labeled with [^{3}H]thymidine (2 μCi/mL) and stimulated with FXa in the absence or presence of a MMP inhibitor, GM 6001 (100 nmol/L), for 24 hours. Media were removed and cells were washed with cold PBS and $HClO_4$ (0.3 mol/L) as previously described.[16] Cells were solubilized by addition of 0.3 mL NaOH (0.1 mol/L) for 30 minutes at 37°C. Aliquots (0.2 mL) were added to 3 mL of scintillant. [^{3}H]Thymidine incorporation was determined by liquid scintillation spectrometry.

Cell Invasion Assay

To determine SMC invasion, a commercially available cell invasion assay kit (Chemicon International) was used.[22] This kit possesses 2 chambers: 1 inner chamber for cell seeding and an outer chamber for cell culture medium. An 8-μm pore size polycarbonate membrane separates the chambers. Invasive cells are able to dissolve the matrix and to migrate through it to the lower surface of the polycarbonate membrane. According to the manufacturer's protocol, 3×10^5 cells were seeded into the inner chamber in serum-free medium. Cells were stimulated with FXa (100 nmol/L) in the absence or presence of the FXa inhibitor DX-9065a (10 μmol/L) or the MMP inhibitor GM 6001 (100 nmol/L). After an incubation period of 6 days, cells from the inner chamber were removed, and the lower surface of the polycarbonate membrane was stained with the solution provided. Cells were photographed and counted using an Olympus Optical microscope BX50 F (Olympus Optical). To standardize the cell count, cells were counted in the central and 4 peripheral microscope fields.

Statistics

Data represent the mean±SEM of n experiments. Statistical analysis was performed using a paired 2-tailed *t* test. Values of $P\leq0.05$ were considered significant.

Results

Pro–MMP-2 and Pro–MMP-9 in Cultured Human SMCs

Under basal conditions, cultured human SMCs expressed pro–MMP-2, which was released into the cell culture medium. Zymography of culture medium from SMCs stimulated with FXa (100 nmol/L) revealed a band of MMP-2. This indicates a cleavage of pro–MMP-2 by FXa. In contrast, FX (100 nmol/L) had no effect on the cleavage of MMP-2 (Figure 1A).

Cells treated with FXa did not express pro–MMP-9 or MMP-9. This indicates that FXa promotes conversion of MMP-2, but not of MMP-9, in cultured human SMCs.

Generation of MMP-2 by FXa was confirmed by Western blotting (Figure 1B). Media of FXa-stimulated cells were

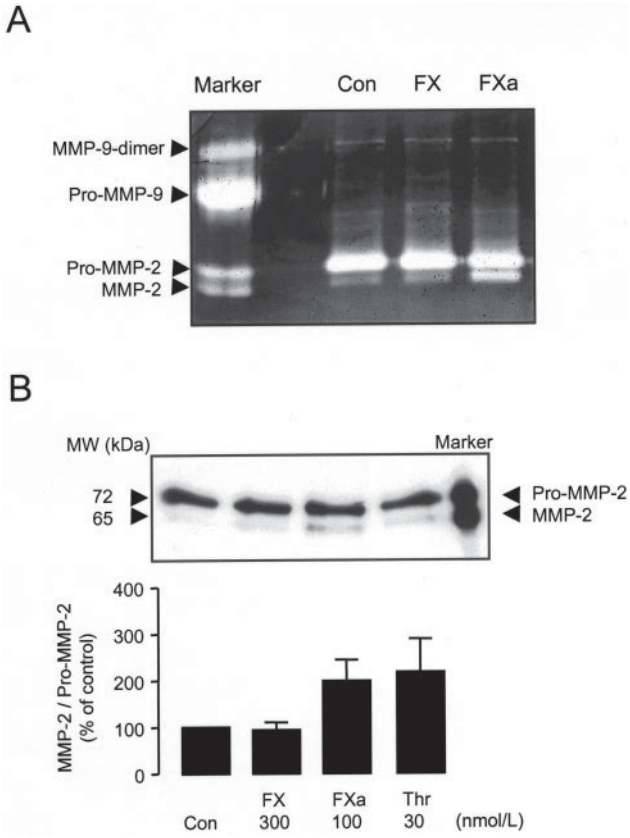

Figure 1. Effects of FX (100 nmol/L) and FXa (100 nmol/L) on pro-MMP and MMP activity (zymography) (A) and protein expression (Western blotting) (B) in conditioned medium from SMCs. A, FXa increased MMP-2 expression but did not stimulate MMP-9; FX had no effects. B, Cleavage of pro–MMP-2 to MMP-2 by FXa and thrombin (30 nmol/L). Pro–MMP-2 and MMP-2 were detected by specific monoclonal antibodies in lyophilized conditioned medium of SMCs, stimulated with FX (300 nmol/L), FXa (100 nmol/L), and α-thrombin (30 nmol/L) for 24 hours. Data are mean±SEM of n=3 to 4 experiments. *$P<0.05$ control (medium from unstimulated cells) vs FXa or thrombin.

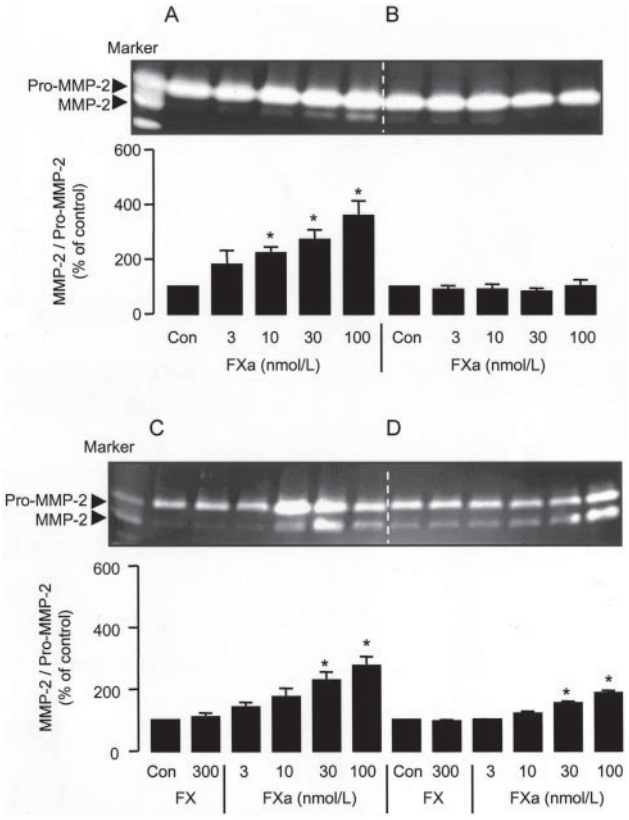

Figure 2. Effects of FXa (3 to 100 nmol/L) on MMP-2 release from stimulated SMCs (A and C) as compared with MMP-2 generation by FXa after addition to cell-free medium (B and D). Medium from stimulated cells was collected either after 1 hour (A and B) or after 72 hours (C and D) and subjected to zymography. There was a considerably stronger stimulation of MMP-2 after its addition to the cells (A and C) than to the cell-free medium (B and D). Data are mean±SEM of n=3 experiments. *$P<0.05$ control (medium from unstimulated cells) vs FXa.

subjected to immunoblotting using a monoclonal antibody specific for pro–MMP-2 and MMP-2. Blots are demonstrating the presence of MMP-2 in the medium of FXa-stimulated cells. Media from thrombin-stimulated cells were used as positive controls.

Conversion of Pro–MMP-2 by FXa: Effects of Cellular Stimulation With FXa Compared With Addition of FXa to Pro–MMP-2 Containing Cell-Free Culture Medium

To investigate whether pro–MMP-2 is cleaved directly by FXa into MMP-2, experiments were carried out in cell-free conditioned medium, containing pro–MMP-2 and compared with cell-containing medium after stimulation with FXa. Samples were analyzed on the same zymography gels (Figures 2A through 2D). After 1 hour of stimulation with FXa (10 to 100 nmol/L), there was a significant MMP-2 generation in the presence of cells but not in conditioned medium in the absence of cells (Figures 2A and 2B). When cell-free medium and cell-containing medium were incubated with FXa (3 to 100 nmol/L) for a longer period of time (72 hours), levels of MMP-2 in the presence of cells were similar. However, there was a significantly increased, although lower, generation of MMP-2 by FXa in conditioned medium without cells (Figures 2C and 2D).

Conversion of Pro–MMP-2 Into MMP-2 and Release of MMP-2 Is Specific for FXa

To investigate whether the conversion of pro–MMP-2 into MMP-2 and its release from SMCs is specific for FXa, the selective FXa inhibitor DX-9065a was used. DX-9065a (0.3 to 10 μmol/L) inhibited the release of MMP-2 by FXa (100 nmol/L) in a concentration-dependent fashion (Figure 3). To exclude the possible involvement of thrombin or plasmin in FXa effects on MMP-2, hirudin and aprotinin were used. Neither hirudin (10 to 300 nmol/L) nor aprotinin (0.1 to 10 μmol/L) did affect FXa-mediated conversion of pro–MMP-2 into MMP-2 (data not shown). Additionally, when the effects of FXa were studied in cell-free medium, DX-9065a inhibited the conversion of pro–MMP-2 into MMP-2 in a concentration-dependent fashion (data not shown). These data demonstrate that FXa specifically releases MMP-2 and converts pro–MMP-2 into MMP-2, and that this action does not involve the serine proteases thrombin or plasmin.

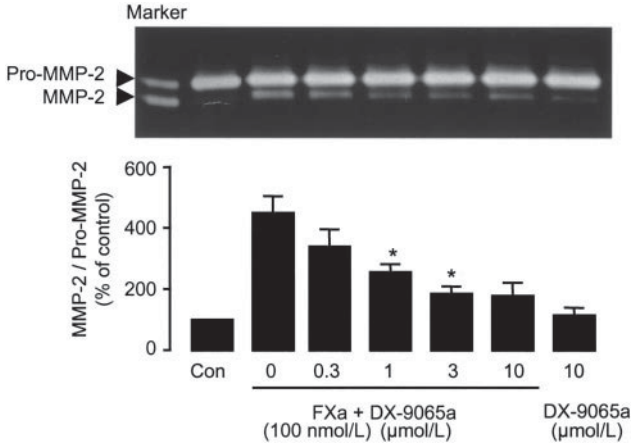

Figure 3. Inhibition of FXa (100 nmol/L)-induced release of MMP-2 from SMCs by the specific FXa inhibitor DX-9065a (0.3 to 10 μmol/L) as detected by zymography. DX-9065a alone (10 μmol/L) had no effect. Data are mean±SEM of n=3 experiments. *$P<0.05$ FXa vs FXa+DX-9065a.

Effects of FXa on Arterial SMCs

To establish that the stimulatory effects of FXa on MMP-2 generation are not restricted to SMCs from venous tissue, additional experiments were carried out in arterial SMCs. Stimulation of human mammary artery SMCs with FXa (30 to 100 nmol/L) also resulted in generation of MMP-2. This conversion of pro–MMP-2 into MMP-2 was inhibited by DX-9065a (1 μmol/L), but not by hirudin (1 μmol/L) or aprotinin (1 μmol/L) (Figure 4). This confirmed the findings on venous SMCs.

Effects of FXa on MMP-2 in SMC Lysates

SMCs were stimulated with FXa (3 to 100 nmol/L) and afterward the lysates were analyzed by zymography. Data show various bands of cell-bound gelatinolytic activity (Figure 5). A strong band of pro–MMP-2 was determined. Furthermore, a weaker band of an intermediate form of MMP-2 (68 kDa) in addition to active MMP-2 (65 kDa) was seen. On stimulation with FXa, an increased formation of intermediate MMP-2 was observed at 30 to 100 nmol/L FXa (Figure 5). This indicates the contribution of cellular mechanisms to the activation of MMP-2 in cultured human vascular SMCs by FXa.

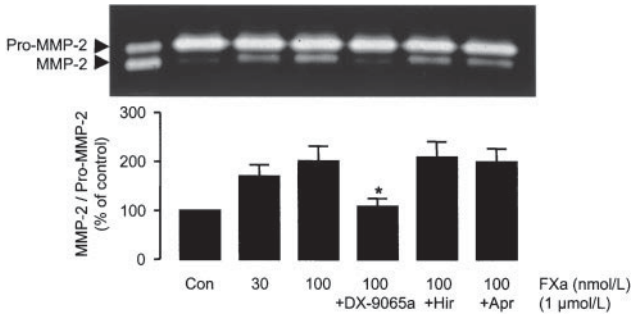

Figure 4. Effects of FXa (100 nmol/L) on the release of MMP-2 from SMCs derived from the human mammary artery as detected by zymography. DX-9065a (1 μmol/L) inhibited FXa-induced conversion of pro–MMP-2 to MMP-2 nearly completely, whereas hirudin (1 μmol/L) or aprotinin (1 μmol/L) had no effects. Data are mean±SEM of n=3 experiments. *$P<0.05$ FXa (100 nmol/L) vs FXa+DX-9065a.

FXa-Induced DNA Synthesis Is Reduced by MMP Inhibition

To investigate whether FXa-induced MMP-2 activation contributes to proliferation of human vascular SMCs,[14] mitogenesis was measured by [^{3}H]thymidine incorporation into cellular DNA. FXa (100 nmol/L) increased the incorporation of [^{3}H]thymidine 4- to 5-fold above control. This strong mitogenic effect of FXa was significantly inhibited by the specific FXa inhibitor DX-9065a.[16] Inhibition was also seen by preincubation of cells with the MMP inhibitor GM 6001 (100 nmol/L) (Figure 6). Neither DX-9065a[16] nor GM 6001 alone affected cellular [^{3}H]thymidine incorporation.

FXa-Induced Extracellular Matrix Invasion Is Reduced by MMP Inhibition

A cell invasion assay was used to investigate whether FXa-induced release of active MMP-2 mediates ECM invasion of SMCs. Cells were stimulated for 6 days with FXa (100 nmol/L) in the absence or presence of either DX-9065a (10 μmol/L) or GM 6001 (100 nmol/L). Microscopy revealed that FXa stimulated SMC migration through the matrix gel. Pretreatment of cells with either the FXa inhibitor DX-9065a or the MMP inhibitor GM 6001 reduced these effects back to baseline. Treatment of cells with the inhibitors alone had no effect on cell invasion (Figure 7).

Discussion

The activation of MMPs plays a significant role in both physiological and pathophysiological conditions.[1–4] Therefore, mechanisms of activation are of great interest in the understanding of ECM regulation. In the present study, we report that the coagulation factor Xa stimulates the release of MMP-2 in human SMCs. In addition, conversion of pro–MMP-2 into MMP-2 is also increased in cell-free medium containing pro–MMP. Similar results were obtained on human arterial SMCs and human fibroblasts (Figure 4, data for fibroblasts are not shown). These findings provide new insights into the mechanism(s) SMC proliferation and matrix invasion. In addition to the role of FXa in blood coagulation, this represents a novel function of FXa, which has not been described in SMCs so far.

It is well established that most MMPs are secreted as inactive proenzymes and are activated in the extracellular space. Activation is caused by disruption of the Zn^{2+}-blockade in the catalytic domain, excreted by the cysteine residue in the propeptide domain.[2,4] Our findings indicate that the serine protease FXa initiates the process of pro–MMP-2 cleavage, eventually resulting in its release into the extracellular space. Cleavage of pro–MMP-2 and generation of MMP-2 was demonstrated by gelatin zymography (Figure 1A) and Western blotting using monoclonal antibodies specific for pro–MMP-2 (72 kDa) and active MMP-2 (65 kDa) (Figure 1B). There was no detectable pro–MMP-9 or MMP-9 by zymography after stimulation of the cells with FXa. These findings suggest that FXa preferentially modifies the MMP-2 pathway but does not interfere with MMP-9 in human SMCs.

Next, it was investigated whether the active MMP-2 in the medium was derived from extracellular cleavage of the secreted proenzyme and/or whether cellular mechanisms

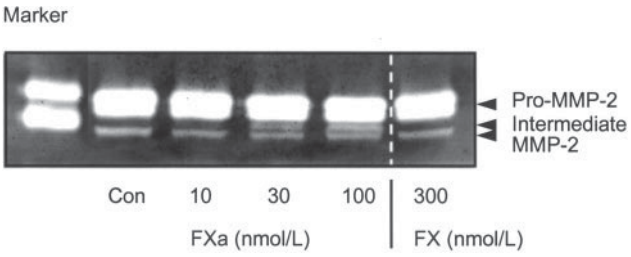

Figure 5. Effects of FXa (10 to 100 nmol/L) on MMP-2 in cell lysates of cultured venous SMCs as detected by zymography. Pro-, intermediate, and active MMP-forms were detected according to the different molecular size. Appearance of an intermediate MMP-2 form was observed after stimulation with FXa (30 to 100 nmol/L). Representative zymography of n=3 independent experiments.

were involved. In these experiments, FXa was added either to the SMCs with subsequent collection of the medium or directly to the cell-free supernatants of SMCs containing secreted pro–MMP-2. After short-term incubation (1 hour), there was only generation of MMP-2 after stimulation of cells. At longer incubation periods (72 hours), this effect was maintained. In cell-free medium, stimulated with FXa, a significantly increased level of MMP-2 was now detected (Figure 2). This amount of MMP-2 could be considerably further enhanced by increasing the concentration of FXa in the medium above 100 nmol/L (data not shown). This phenomenon might be explained by the ability of active MMPs to stimulate additional lytic enzymes[1,4] or simply a longer duration of action might explain the stronger effects seen after longer incubation periods. In any case, the conversion of pro–MMP-2 into MMP-2 by FXa was specific for FXa because it was concentration-dependently inhibited by DX-9065a, a specific active-site inhibitor of FXa[23,24] (Figure 3). Furthermore, the possibility that thrombin or plasmin may have contributed to FXa-induced activation of MMP-2 was excluded because incubation of SMCs with hirudin and aprotinin had no effect (Figure 4).

In SMC lysates, we detected pro–MMP-2 (72 kDa), an intermediate form (68 kDa), and active MMP-2 (65 kDa) after stimulation with FXa (Figure 5). These findings are in concert with previous reports of multimer forms of MMP-2 in lysates

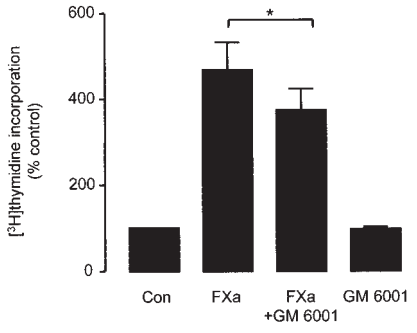

Figure 6. [^{3}H]Thymidine incorporation in cultured venous SMCs. FXa (100 nmol/l)-induced [^{3}H]thymidine incorporation was inhibited by the MMP inhibitor GM 6001 (100 nmol/L); GM 6001 on its own had no effects. Data are mean±SEM of n=8 experiments performed in triplicate. *$P<0.05$ FXa vs FXa+MMP-inhibitor.

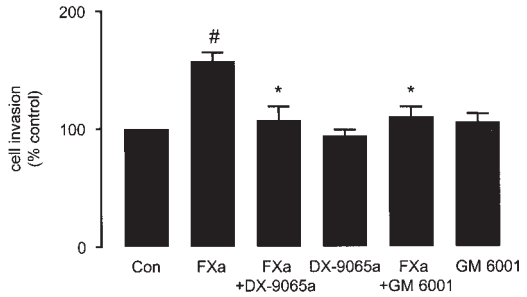

Figure 7. FXa (100 nmol/L)-stimulated venous SMC matrix invasion and its inhibition by DX-9065a (10 μmol/L) or GM 6001 (100 nmol/L). No direct effects of DX-9065a or GM 6001 were observed. Data are mean±SEM of n=5 to 6 experiments. #$P<0.05$ FXa vs control (unstimulated SMCs); *$P<0.05$ FXa vs FXa+inhibitors.

from various cell types.[18,26–28] At 30 to 100 nmol/L FXa, we observed the appearance of an intermediate form of MMP-2 (Figure 5), indicating a FXa-dependent pro–MMP-2 cleavage via an intermediate cell-associated complex. Although these data are probably due to complex mechanisms, they might suggest that FXa-stimulated conversion of pro–MMP-2 to MMP-2 is a membrane-related process.[26,29] Preliminary studies with separated membrane and cytosolic fractions of SMCs appear to support this conclusion (data not shown).

It has been reported that MMP-2 is activated on the cell surface by MT1-MMP[1,29] and the uPA/plasmin system.[6,30] Thrombin can MT-MMP-dependently activate pro–MMP-2.[18] It is possible that FXa initiates the release of active MMP-2 from the cell surface by altering the MT1-MMP/TIMP-2/MMP-2 complex. This hypothesis is supported by the findings that TIMP-2 binds and inhibits active MMP-2 and that the MMP-2/TIMP-2 complex is then released from the cell surface.[25] Another study reported the release of active MMP-2 from the cell surface and the control of integrin-mediated MMP-2 activation by collagen.[26] The FXa-induced release of MMP-2 was not affected in cells pretreated with TIMP-2 (2 to 20 nmol/L) (not shown).

Our observations raise the question whether metalloproteinases contain cleavage sites for FXa, which could be located in the propeptide domain to induce MMP activation. A potential FXa cleavage site[31] in the amino acid sequence of MMP-2[32] is present at the border between the propeptide domain and the first catalytic domain and might be responsible for pro–MMP-2 activation. However, the exact mechanism by which pro–MMP-2 is converted to active MMP-2 is still unknown.[26]

The functional significance of MMP-2, aside from degrading ECM, is the regulation of cell proliferation, migration, and tumor invasion.[10,13,33,34] In addition, an important role of MMP-2 in the regulation of SMC proliferation, migration, and matrix invasion has been reported.[11,12,35,36] It has been demonstrated that FXa stimulates SMC proliferation[16] and that the synthetic FXa inhibitor DX-9065a reduces SMC proliferation in vitro[16] and in vivo.[24] We hypothesize that FXa-induced MMP-2 activation may contribute to mitogenesis of SMCs and matrix invasion. Both effects were are reduced by inhibitors of FXa and MMPs, respectively. Therefore, we conclude that FXa contributes via MMP activation to both cell proliferation and SMC matrix invasion.

These findings might be of clinical importance for newly developed FXa inhibitors. Several compounds are currently subject of clinical trials, for example in patients with acute coronary syndrome.[37] In addition to the anticoagulatory effect, an inhibition of FXa-induced MMP activation by these compounds might contribute to the patients' benefit.

In summary, we demonstrate for the first time that FXa generates significant levels of MMP-2 in the environment of SMCs by stimulation of MMP-2 release and conversion of pro–MMP-2 to MMP-2. These increased local levels of MMP-2 may play a role in FXa-induced cell proliferation and matrix invasion.

Acknowledgments

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Arbeit #2

Van den Boom M, Sarbia M, von Wnuck Lipinski K, Mann P, Meyer-Kirchrath J, Rauch BH, Grabitz K, Levkau B, Schrör K, Fischer JW.

Differential regulation of hyaluronic acid synthase isoforms in human saphenous vein smooth muscle cells: Possible implications for vein graft stenosis.

Circ Res 2006; 98:36-44.

Differential Regulation of Hyaluronic Acid Synthase Isoforms in Human Saphenous Vein Smooth Muscle Cells

Possible Implications for Vein Graft Stenosis

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Abstract—Autologous saphenous vein bypass grafts (SVG) are frequently compromised by neointimal thickening and subsequent atherosclerosis eventually leading to graft failure. Hyaluronic acid (HA) generated by smooth muscle cells (SMC) is thought to augment the progression of atherosclerosis. The aim of the present study was (1) to investigate HA accumulation in native and explanted arterialized SVG, (2) to identify factors that regulate HA synthase (HAS) expression and HA synthesis, and (3) to study the function of the HAS2 isoform. In native SVG, expression of all 3 HAS isoforms was detected by RT-PCR. Histochemistry revealed that native and arterialized human saphenous vein segments were characterized by marked deposition of HA in association with SMC. Interestingly, in contrast to native SVG, cyclooxygenase (COX)-2 expression by SMC and macrophages was detected only in arterialized SVG. In vitro in human venous SMC HAS isoforms were found to be differentially regulated. HAS2, HAS1, and HA synthesis were strongly induced by vasodilatory prostaglandins via G_s-coupled prostaglandin receptors. In addition, thrombin induced HAS2 via activation of PAR1 and interleukin 1β was the only factor that induced HAS3. By small interfering RNA against HAS2, it was shown that HAS2 mediated HA synthesis is critically involved in cell cycle progression through G_1/S phase and SMC proliferation. In conclusion, the present study shows that HA-rich extracellular matrix is maintained after arterialization of vein grafts and might contribute to graft failure because of its proproliferative function in venous SMC. Furthermore, COX-2–dependent prostaglandins may play a key role in the regulation of HA synthesis in arterialized vein grafts. (***Circ Res*****. 2006;98:36-44.)**

Key Words: hyaluronic acid ■ extracellular matrix ■ cyclooxygenase-2 ■ vein graft stenosis

Autologous saphenous vein grafts (SVG) are frequently used for bypass grafting in patients with symptomatic occlusive disease of coronary arteries or arteries of the lower extremities. Subsequently, the grafted vein segments are exposed to arterial blood pressure and shear stress, which are thought to initiate intensive remodeling, intimal thickening, in-graft thrombosis, and superimposed atherosclerosis associated with long-term failure rates of approximately 30% to 40%.[1,2] The pathophysiological mechanisms eventually resulting in graft failure include activation and dedifferentiation of vascular smooth muscle cells (SMC) from a contractile into a secretory phenotype characterized by high migratory and proliferative activity.[3] In addition, the extracellular matrix (ECM) undergoes remodeling in arterialized venous grafts. This remodeling is characterized by high ECM turnover conferred by matrix metalloproteinases 1, -2, and -9[4,5] and increased deposition of newly synthesized ECM components including collagen and proteoglycans.[6,7] ECM remodeling is thought to be required for the proliferative and migratory activation of SMC and to support intimal volume expansion.[8]

Recently, hyaluronic acid (HA) has been shown to be a major component of thickened neointimal, restenotic, and atherosclerotic lesions in humans and to be associated with proliferating SMC and thrombosis of eroded plaques,[9,10] suggesting that HA is a critical factor during the pathophysiology of cardiovascular disease. HA is a polysaccharide composed of repeating disaccharide units (D-glucuronic acid β-1,3-*N*-acetylglucosamine-β1,4) that is synthesized at the plasma membrane by 3 different HA synthases (HAS1 to -3). During synthesis the growing HA-polymer is extruded into the extracellular environment.[11] Studies in mesothelial cells, epithelial cells, and endothelial cells showed that epidermal growth factor, platelet-derived growth factor (PDGF)-BB, and transforming growth factor (TGF)-β1 all participate in transcriptional regulation of HAS isoforms in both an isoform

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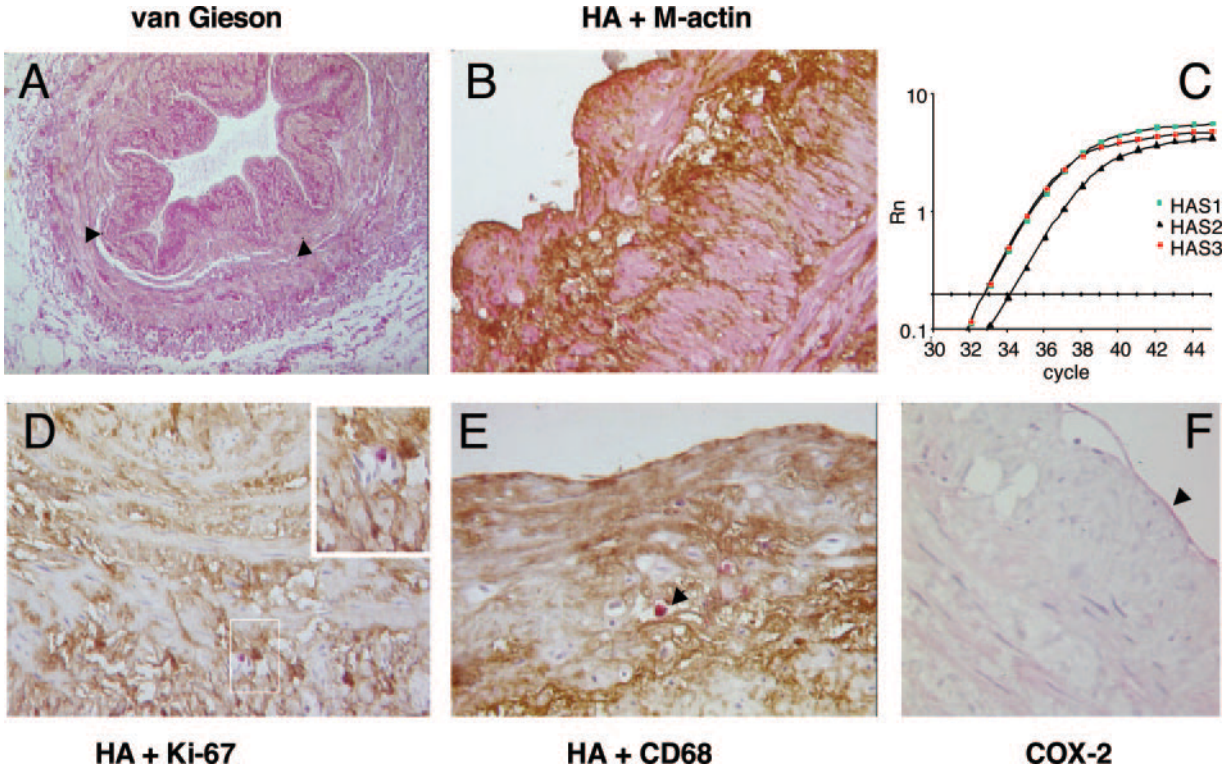

Figure 1. Representative staining of a native human saphenous vein from the lower limb of a 62-year-old woman. A, Cross-section showing SMC (light brown) in the intima and in the media and diffuse thickening and collagenous fibrosis (red) of the intima (elastic–van Gieson stain; original magnification: ×63). The border between media and intima is indicated by arrow heads. B, Combined intimal staining for HA using HA-binding protein (brown) and M-actin (red) (original magnification: ×200). C, Representative real-time RT-PCR for HAS1, HAS2, and HAS3 performed from RNA that was isolated from a native human saphenous vein segment. D, Double staining of HA (brown) and Ki-67 (red) (original magnification: ×200); inset shows a proliferating cell at 400-fold magnification. E, Combined staining of HA (brown) and CD68 (red); arrow head indicates a CD68 positive macrophage; original magnification: ×200. F, Immunohistochemical staining for COX-2 (red) (original magnification: ×200); arrow head indicates the COX-2–positive endothelium.

and cell type–specific manner.[12–14] However, very little is known about the regulation of HAS isoform expression in vascular SMC. HAS2, which is the main HAS isoform in cultured vascular SMC,[15] is induced by PDGF-BB[15] and vasodilatory prostaglandins.[16] In vitro studies using vascular SMC and fibroblasts showed that HA is critically involved in proliferation and migration[15,17,18] and cell spreading.[16] Thus, based on the evidence from various forms of arterial vessel disease and functional studies in cultured SMC, it is likely that HA plays an important role during neointimal thickening and possibly also during failure of venous bypass grafts.

The aims of the present study were (1) to analyze native saphenous vein segments and arterialized SVG with respect to HA-accumulation, SMC proliferation, and macrophage accumulation; (2) to study the transcriptional regulation of the 3 HAS isoforms in human venous SMC; and (3) to investigate the functional significance of HAS2-mediated HA synthesis by small interfering RNA (siRNA) targeting HAS2 in vitro.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture

Venous SMC were isolated by the explant technique from the media of human saphenous veins. Leftover segments were obtained from patients undergoing coronary bypass surgery according to the guidelines of the local Medical Ethical Board. SMC of passages 4 to 10 were used. Four different venous cell lines were studied. The SMC were grown in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. Cells were seeded at 10 000 cells/cm^2, growth arrested by serum withdrawal for 24 hours, and subsequently treated with the compounds to be studied.

Analysis of Human Veins

Native Vein Segments

Leftover fragments of surgical specimens from saphenous veins prepared for bypass grafting (n=12; 4 females, 8 males; median age, 64 years [range, 37 to 82 years]) were collected prospectively from the files of the Institute of Vascular surgery (Universitätsklinikum Düsseldorf). The veins were cut transversally, fixed in 10% formalin, and embedded in paraffin. Four-micrometer sections from the paraffin blocks were stained with hematoxylin/eosin and elastic–van Gieson stain. The staining patterns were evaluated by a senior pathologist (M.S.).

Veins Exposed to Arterial Blood Pressure

Six human veins that had been used to bypass arterial stenosis were obtained from the files of the Institute of Pathology (Universitätsklinikum Düsseldorf). Five veins had been implanted in the lower extremity and 1 in the upper extremity. Explantation had been performed in the year 2004 for various reasons: in 3 cases, the lower extremity had been amputated for ischemic soft tissue necrosis; in 1 case, the venous bypass was selectively resected because of thrombotic occlusion; in 1 case, for obliteration of the *Arteria brachialis*; and in 1 case, for unknown reasons. Two of the 6 patients were

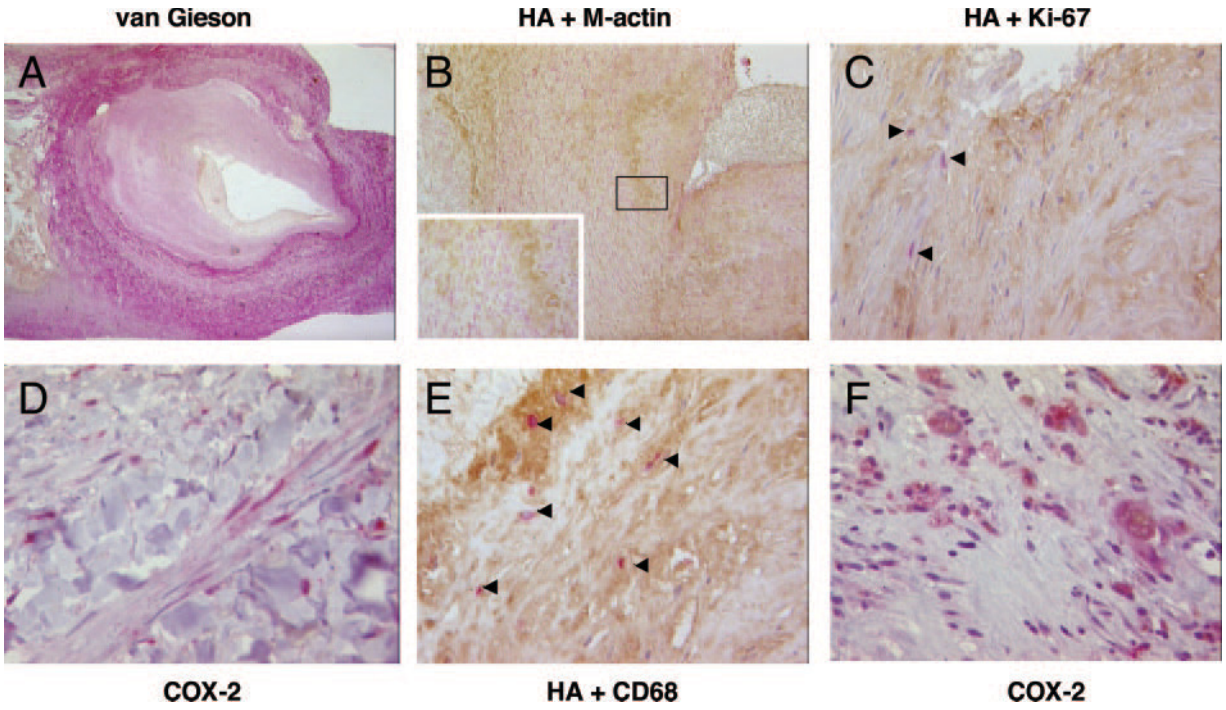

Figure 2. Representative staining of an arterialized human vein graft (femoro-popliteal bypass) from the lower extremity of a 79-year-old woman. A, Cross-section showing marked collagenous fibrosis (red) and muscular atrophy of the media (elastic–van Gieson stain; original magnification: ×63) and marked excentric thickening and fibrosis of the intima with concomitant stenosis of the lumen. B, Double staining for HA (brown) and M-actin (red) (original magnification: ×200). The inset provides a higher magnification of the framed area (×400). C, Double staining for HA and the proliferation marker Ki-67 (red) (original magnification: ×200); arrow heads mark proliferating cells. D, Double staining for HA (brown) and CD68 (red) showing a number of macrophages in the wall of this vein (original magnification: ×200). E, Strong COX-2 expression in medial smooth muscle cells (indicated by spindle shape of cells and nuclei; original magnification: ×200). Compare with Figure 1F. F, Section derived from a thrombosed, arterialized vein graft. Accumulation of COX-2–positive cells, which are mostly macrophages, as indicated by the rounded shape and the brownish granular pigment, which was hemosiderin pigment.

female, the age ranged between 41 and 79 years (median, 57 years). Veins were processed as described above.

Results

Native Saphenous Veins

Figure 1A shows a representative section of a native saphenous vein derived from a 62-year-old women, which was prepared for bypass surgery. The thickened intima and the tunica media contained muscle actin (M-actin)–positive cells (Figure 1B). In the intima, media, and adventitia, abundant accumulation of HA was detected, which colocalized intimately with M-actin–positive cells in the intima and in the media (Figure 1B). In addition, mRNA expression of all 3 HAS isoforms was detected by RT-PCR (not shown) and real-time RT-PCR (Figure 1C). Double immunohistochemical staining for HA and the Ki-67 antigen, which is only expressed in proliferating cells,[19] revealed only a few proliferating cells in the wall of native veins (Figure 1D). Rare CD68-positive macrophages were detected in both the intima and/or the media of human veins (Figure 1E). COX-2 expression was not detectable in SMC of native SVG, whereas COX-2 reactivity was detected in endothelial cells as an internal control (Figure 1F).

Veins Exposed to Arterial Blood Pressure

Conventional histological examination based on hematoxylin/eosin stains and on elastic–van Gieson stain revealed various degrees of intimal thickening resulting in moderate up to subtotal stenosis of the lumen (Figure 2A). All specimens showed fibrosis of the media, which was very pronounced in 4 cases. The thickened intimas were characterized by accumulation of SMC, which were intimately associated with HA (Figure 2B). SMC showed a very low proliferative activity as indicated by Ki-67 staining (Figure 2C). In 2 of the explanted SVG, thrombosis was observed. In the 2 thrombosed SVG and in 1 of the cases without thrombosis, strong expression of COX-2 in intimal and medial SMC was observed (Figure 2D). In the cases with thrombosis, marked infiltration of the intima and subintimal media with CD68-positive macrophages was observed (Figure 2E and 2F), which strongly expressed COX-2 (Figure 2F). In contrast, in the cases without thrombosis, low numbers of CD68-positive macrophages were present in both the intima and media.

Differential Regulation of HAS1 and HAS3 Isoforms in Human Venous SMC

The mRNA of HAS1, which is the HAS isoform expressed at the lowest level in cultured human SMC,[15] was induced only by the prostacyclin (PGI_2) analog iloprost and prostaglandin E_2 (PGE_2) (Figure 3). To identify the responsible prostaglandin receptors, selective receptor agonists were used. Both the specific PGI_2 (IP)-receptor agonist cicaprost[20] and the specific E-type prostaglandin receptor subtype 2 (EP_2-receptor) agonist butaprost[21] mimicked the effects of iloprost and

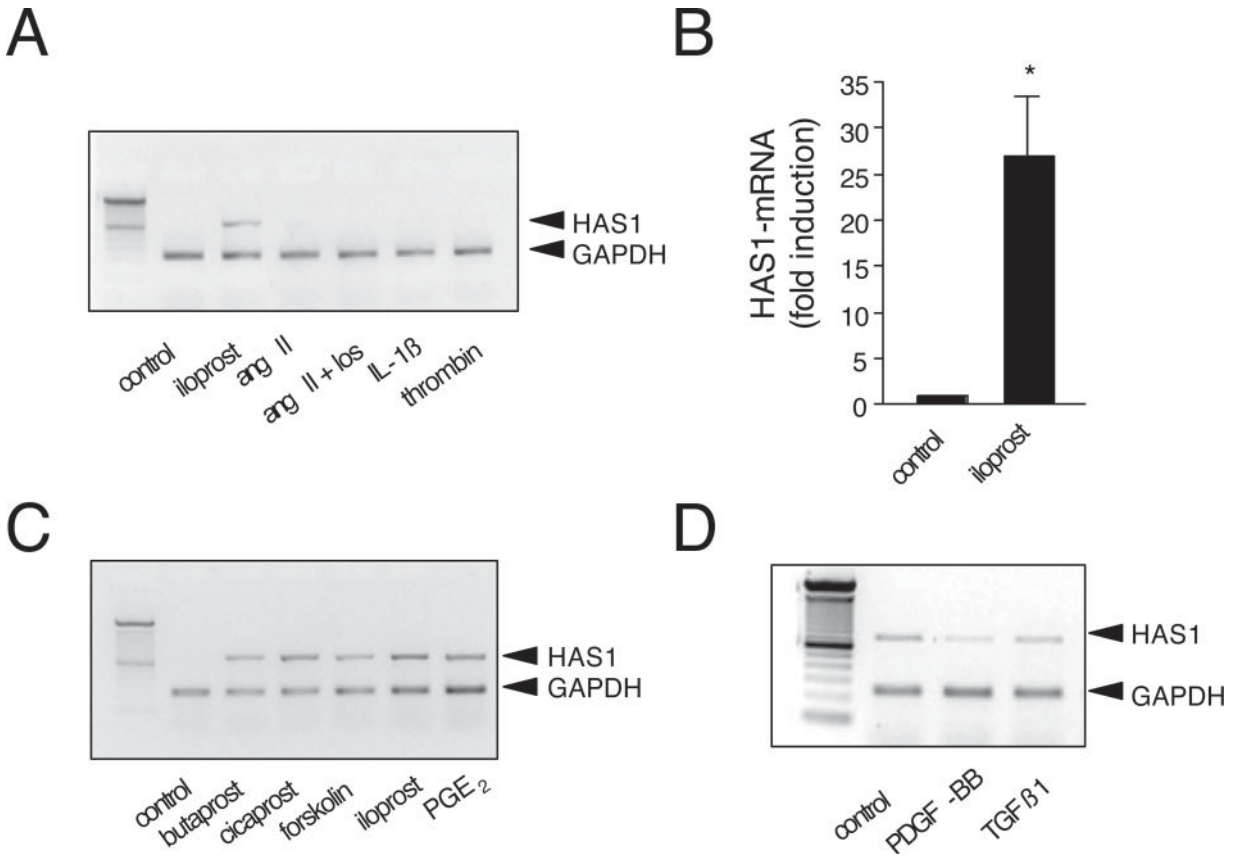

Figure 3. Characterization of HAS1 expression in human venous SMC in vitro. A, After 24 hours of serum withdrawal, cells were stimulated for 3 hours with iloprost (100 nmol/L), angiotensin II (10^{-7} mol/L), angiotensin II (10^{-7} mol/L) plus losartan (10^{-5} mol/L), IL-1β (10 ng/mL), and thrombin (10 U/mL). Subsequently semiquantitative RT-PCR for HAS1 was performed. B, Densitometric quantification of the iloprost effect, which was expressed as fold over control after calculation of the ratio of HAS1 and GAPDH bands; n=3 independent experiments (mean±SEM). *$P<0.05$ vs control. C, HAS1 expression after incubation of venous SMC with butaprost (1 μmol/L), cicaprost (100 nmol/L), forskolin (10 μmol/L), iloprost (100 nmol/L), and PGE_2 (100 nmol/L). D, HAS1 expression in response to PDGF-BB and TGF-β1. In A, C, and D, original gels representative of n=3 experiments are shown.

PGE_2, suggesting that the G_s-coupled IP- and EP_2 receptors are both capable of inducing HAS1 mRNA expression (Figure 3) in venous SMC. In line with the hypothesis that cAMP-dependent signaling of the G_s-coupled IP and EP_2 receptors[22] is responsible for HAS1 induction, forskolin, an activator of adenylate cyclase, induced HAS1 as well (Figure 3C). PDGF-BB, TGF-β1, angiotensin-II, thrombin, and interleukin (IL)-1β had no effect on HAS1 expression (Figure 3). Angiotensin II (10^{-7} mol/L) was used in the presence and absence of the AT_1 receptor antagonist losartan (10^{-5} mol/L), because blocking of the AT_1 receptor can be used to unmask effects of the AT_2 receptor that could otherwise be missed. However, angiotensin II did not have any effects on HAS isoform expression under the current experimental conditions.

HAS3 expression was analyzed in response to the same factors as mentioned above. Interestingly, HAS3 was markedly induced only by IL-1β (Figure 4).

Differential Regulation of HAS2 in Cultured Venous SMC

The mRNA of HAS2, which is the main isoform in venous SMC,[15] was induced by 100 nmol/L iloprost and 10 U/mL thrombin, respectively (Figure 5A and 5B). The HAS2 induction in response to thrombin was concentration dependent starting at 1 U/mL (data not shown) and could be mimicked by the PAR1 activating peptide (AP)-1 (100 μmol/L) as shown in Figure 5C. In contrast, the PAR2, -3, and -4 activating peptides had no significant effect on HAS2 expression. Furthermore, HAS2 was strongly induced by PDGF-BB (20 ng/mL), as described previously,[15] and was not changed by TGF-β1 (10 ng/mL).

The regulation of HAS2 by prostaglandins was investigated in more detail, because prostaglandin-mediated HAS2 mRNA upregulation was strongest among the stimuli investigated and dramatic upregulation of COX-2 was detected in half of the cases of explanted, arterialized venous bypass grafts (Figure 2). The mRNA induction of HAS2 was maximal at 3 and 6 hours after stimulation and occurred in a concentration-dependent manner (data not shown). HAS2 mRNA was also strongly upregulated by a selective EP_2 agonist (butaprost), forskolin, and dibutyryl-cAMP (db-cAMP), suggesting that G_s-coupled IP and EP_2 receptors were involved (Figure 6A). Consequently stimulation of venous SMC with iloprost, cicaprost, and PGE_2 resulted in increased secretion and accumulation of HA in the conditioned cell culture medium (Figure 6B). Furthermore, prostaglandin-induced HA secretion was mimicked by forskolin and db-

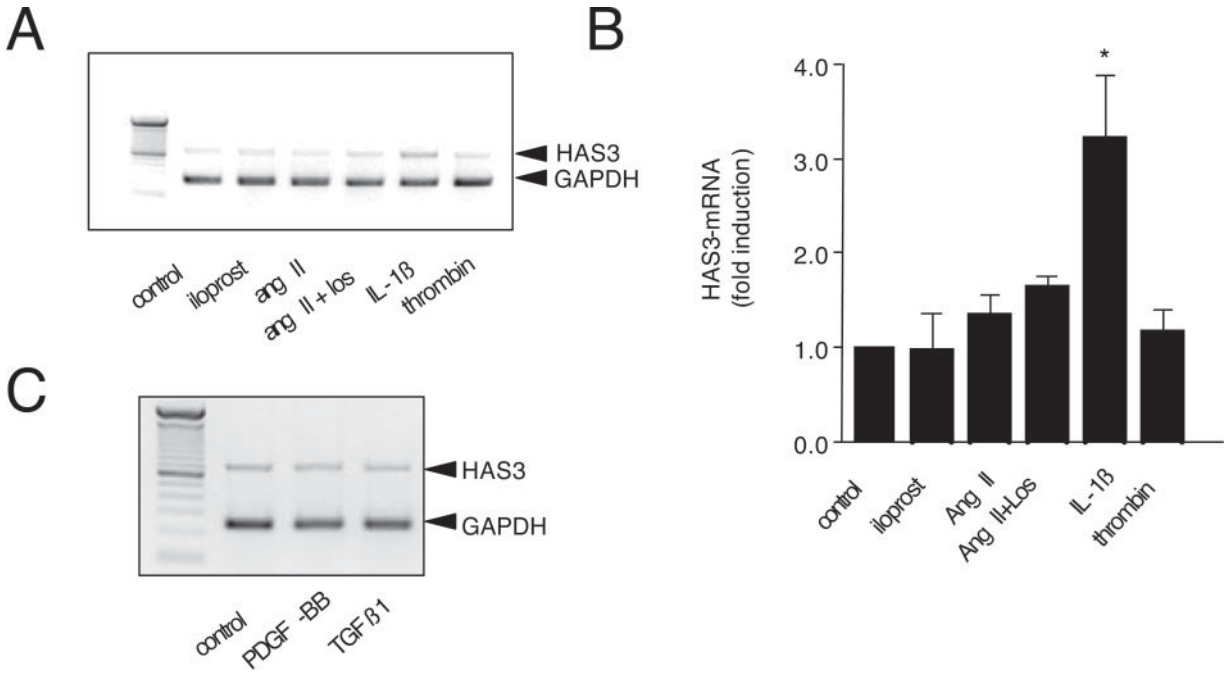

Figure 4. Characterization of HAS3 expression in human venous SMC in vitro. A, After 24 hours of serum withdrawal, cells were stimulated for 3 hours with iloprost (100 nmol/L), angiotensin II (10^{-7} mol/L), angiotensin II (10^{-7} mol/L) plus losartan (10^{-5} mol/L), IL-1β (10 ng/mL), and thrombin (10 U/mL) and semiquantitative RT-PCR for HAS3 was performed. B, Densitometric quantification expressed as fold over control after calculation of the ratio of HAS3 and GAPDH bands; n=3 independent experiments (mean±SEM). *$P<0.05$ vs control. C, HAS3 expression in response to PDGF-BB and TGF-β1. In A and C, original gels of n=3 experiments are shown.

cAMP (not shown) and was inhibited by the protein kinase A (PKA) inhibitor H89 (Figure 6C). Prostaglandin-induced HA synthesis was in the same order of magnitude as after stimulation with 20 ng/mL PDGF-BB (not shown).

Because the present data revealed that prostaglandins induce both HAS1 and HAS2 mRNA, siRNA targeting HAS2 was used to roughly estimate the relative contribution of HAS1 and HAS2 to prostaglandin-induced HA synthesis. HAS2 siRNA significantly inhibited the iloprost-induced expression of HAS2 mRNA by 47.6±4.6% (n=4, $P<0.05$). Furthermore, HAS2 siRNA inhibited the induction of HA secretion by iloprost by 39.3% (mean of 2 independent experiments), which suggests that HAS2 synthesized the majority of HA in response to iloprost.

Induction of Pericellular HA in Human Venous SMC In Vitro

To investigate whether the factors that were shown above to increase the expression of HAS isoforms were also able to induce cell-associated HA, cultured venous SMC were stimulated with vasodilatory prostaglandins (iloprost [100 nmol/L], PGE_2 [100 nmol/L]), with PDGF-BB (20 ng/mL), thrombin (10 U/mL), and IL-1β (10 ng/mL). After 24 hours of serum withdrawal, cells were incubated with streptomyces hyaluronidase to remove any preformed extracellular HA. Subsequently, SMC were stimulated with the factors indicated above for 48 hours and pericellular HA was visualized by HA-binding protein staining. All stimuli induced deposition of pericellular HA. However, deposition of cell-associated HA was stronger in response to PDGF-BB, IL-1β, and thrombin as compared with vasodilatory prostaglandins (online data supplement). In contrast, the stimulated secretion of free soluble HA into the cell culture medium and induction of HAS2 in response to iloprost was at least as high as in response to PDGF-BB and thrombin. This finding suggests that the effects of vasodilatory prostaglandins differ from PDGF-BB, thrombin, and IL-1β with respect to the ratio of secreted and pericellular HA, which might be attributable to differential regulation of additional proteins required for the formation of pericellular HA coats.[23]

Functional Significance of HAS2-Mediated HA Synthesis in Venous SMC

The stimuli used to characterize the differential regulation of HAS isoform expression in venous SMC were selected because of their potential relevance during vein graft stenosis and failure. HAS2 was the HAS isoform that was found to be regulated by most of the stimuli namely the PGI_2 analogue (iloprost), PGE_2, PDGF-BB, and thrombin and contributed most of the HA in response to iloprost. Therefore, the functional significance of HAS2 was investigated, applying HAS2 siRNA to inhibit expression of HAS2 in cultured venous SMC. Cell cycle analysis of SMC after siRNA targeting of HAS2 revealed a partial (≈20%) suppression of the progression through the G_1/S phase 18 hours after PDGF-BB stimulation compared with control siRNA (Figure 7A). Western blot analysis of cell cycle proteins showed marked downregulation of cyclins A and E as well as upregulation of the cyclin-dependent kinase inhibitor p27 after PDGF-BB in cells transfected with HAS2 siRNA compared with control siRNA (Figure 7B). No differences were observed in cyclin D1, p21, and cdk2 (data not shown). Furthermore, HAS2 siRNA caused decreased DNA synthesis in response to PDGF-BB as determined by [^{3}H]-thymidine

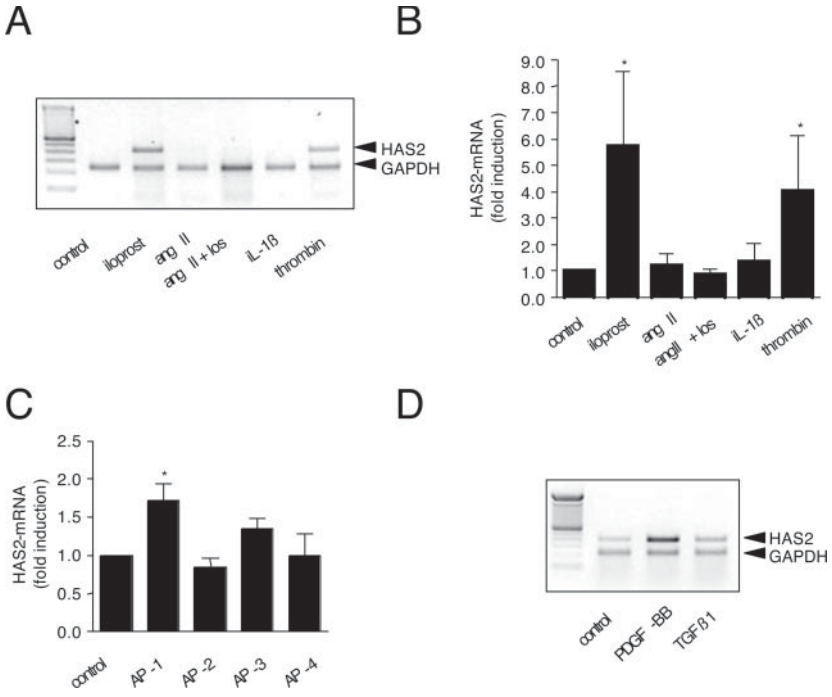

Figure 5. Characterization of HAS2 expression in human venous SMC in vitro. A, After 24 hours of serum withdrawal, cells were stimulated for 3 hours with iloprost (100 nmol/L), angiotensin II (10^{-7} mol/L), angiotensin II (10^{-7} mol/L) plus losartan (10^{-5} mol/L), IL-1β (10 ng/mL), and thrombin (10 U/mL) and semiquantitative RT-PCR for HAS2 was performed. B, HAS2 expression is expressed as fold over control after calculation of the ratio of HAS2 and GAPDH bands after densitometric quantification; n=5 independent experiments (mean±SEM). *$P<0.05$ vs control. C, HAS2 expression after incubation (3 hours) of venous SMC with AP-1 (100 μmol/L), AP-2 (100 μmol/L), AP-3 (100 μmol/L), and AP-4 (100 μmol/L); n=3 independent experiments (mean±SEM). *$P<0.05$ vs control. D, HAS2 expression in response to PDGF-BB and TGF-β1. In A and D, original gels representative of (n=5 and n=3) independent experiments are shown.

incorporation (Figure 8A). HAS2 siRNA caused decreased mitogenesis as determined by cell counting in comparison to venous SMC transfected with nonsilencing control siRNA (Figure 8B). As described previously,[15] cells undergoing mitosis and cytokinesis after PDGF-BB stimulation had a pronounced pericellular HA coat (data not shown). Proliferating SMC of native and arterialized saphenous vein segments as detected by positive Ki-67 staining appeared to be in direct contact with HA-rich pericellular matrix as well (Figure 8C). A schematic diagram illustrating regulation of HAS1 and HAS2 by prostaglandins and the proproliferative function of HAS2-mediated HA synthesis is depicted in Figure 8D.

Discussion

The current investigation characterizes the ECM of saphenous vein segments used for bypass surgery in patients with ischemic artery disease with respect to HA accumulation. All native veins showed intimal thickening, which is in line with previous reports of pronounced intimal thickening of primary vein graft tissue.[6,24] Strong accumulation of HA in association with SMC was observed in the thickened intima of primary as well as in arterialized saphenous vein segments. Because extracellular HA has a relatively short half-life,[25] the presence of HA in arterialized SVG suggests that HA synthesis is induced after bypass grafting. However, the factors that are responsible for induction and maintenance of the HA-rich ECM in saphenous vein grafts after arterialization are unknown. Therefore, the effects of various factors that are known to be involved in control of SMC proliferation, ECM synthesis, and vascular inflammation were analyzed with respect to HA synthesis and HAS isoform expression in cultured SMC from human saphenous vein. The factors investigated in the present study included vasodilatory prostaglandins (iloprost and PGE_2), thrombin, IL-1β, PDGF-BB, and TGF-β1.

PGI_2 and PGE_2 are vasodilatory prostaglandins that are synthesized by PGI_2 synthase and PGE synthase from PGH_2, which is generated by COX-2. Both, HAS1 and HAS2 were induced by iloprost and PGE_2 via the G_s-coupled IP and EP_2 receptors in a cAMP- and PKA-dependent manner. A similar response to vasodilatory prostaglandins with respect to HAS2 expression was recently demonstrated in arterial SMC.[16]

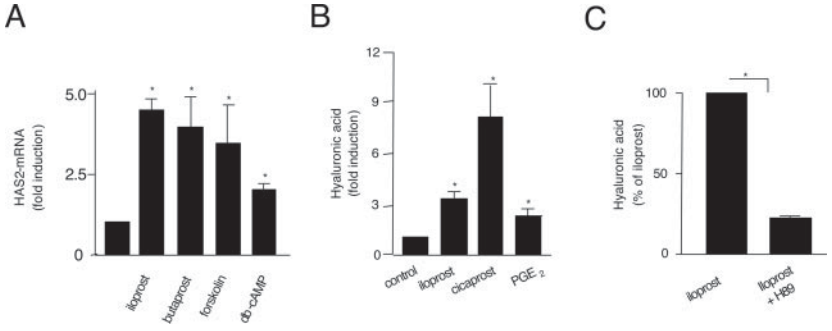

Figure 6. Induction of HAS2 mRNA and HA synthesis by vasodilatory prostaglandins. A, HAS2 expression in venous SMC in response to iloprost (100 nmol/L), butaprost (1 μmol/L), forskolin (10 μmol/L), and db-cAMP (1 mmol/L). HAS2 mRNA expression was determined by semiquantitative RT-PCR after 3 hours and the ratio of the signals of HAS2 and GAPDH were normalized to control. B, Levels of HA in the cell culture medium were determined 24 hours after treatment of SMC with iloprost (100 μmol/L), cicaprost (100 nmol/L), and PGE_2 (100 nmol/L). C, Levels of HA in the cell culture medium 24 hours after treatment of cells with iloprost (100 nmol/L), iloprost+H89 (100 nmol/L). HA levels are presented as percentage of the concentration measured in response to iloprost; n=3, mean±SEM. *$P<0.05$ vs iloprost.

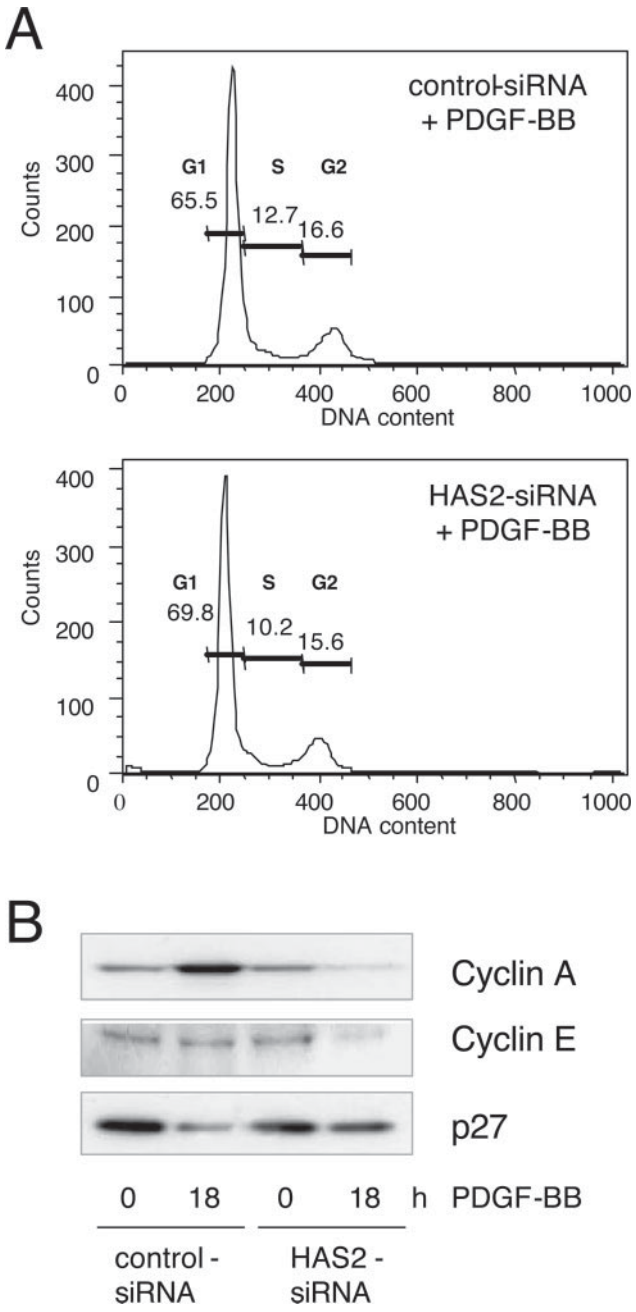

Figure 7. Cell cycle analysis of human venous SMC after siRNA targeting of HAS2 in vitro. A, Twenty-four hours after transfection with nonsilencing control siRNA or HAS2 siRNA, cells were stimulated with PDGF-BB (20 ng/mL) for 18 hours. The cell cycle distribution was analyzed by fluorescence-activated cell sorting, and representative (n=4) DNA profiles are presented. B, Cells were treated as described in A. Expression of cell cycle proteins was determined by Western blot analysis using antibodies against cyclin E, cyclin A, and p21. Representative data of n=3 experiments are shown.

However, this is the first report showing that HAS1 is induced by vasodilatory prostaglandins as well. This finding might be relevant, because strong COX-2 expression was detected in 3 of 6 arterialized veins in the current study, whereas COX-2 was absent from native vein segments. COX-2 is frequently found to be upregulated after vessel injury and during atherosclerosis.[26,27] However, the role of COX-2–dependent prostaglandins during atherosclerosis is currently discussed controversially.[28] PGI_2 mediates vasoprotective and antithrombotic functions by inhibiting platelet aggregation and vasoconstriction, whereas induction of matrix metalloproteinase expression and inflammatory functions have been attributed to PGE_2 in atherosclerotic lesions.[27] Recently, it was shown that transgenic mice overexpressing HAS2 in SMC in apolipoprotein E–deficient mice showed increased atherosclerosis[29] suggesting that HAS2 induction by prostaglandins is proatherogenic. Thrombin is generated in vivo at the lipid surface of activated platelets during thrombus formation which is frequently induced in venous bypass grafts.[30,31] Thrombosis was also detected in 2 of 6 cases in the current study. Thrombin which is known to increase the propensity of SMC to proliferate and migrate[32] activates PAR1, -3, and -4 receptors.[33] Thrombin and the PAR1 activating peptide induced HAS2 expression, suggesting that thrombin via the PAR1 receptor participates in the regulation of HA synthesis in venous SMC.

Monocyte invasion and release of cytokines are early events in vein graft stenosis.[34] HAS3 was induced in response to IL-1β which is a major cytokine released from macrophages. IL-1β was the only stimulus that upregulated HAS3 in human venous SMC. Therefore, it could be hypothesized that HAS3 expression is involved in the inflammatory response induced by macrophages. Notably, it has recently been demonstrated that macrophages adhere to HA-rich structures during inflammatory bowel disease and to intestinal SMC in vitro.[35]

PDGF and TGF-β1 are considered key mediators during medial and intimal thickening of autologous vein grafts[36] and have been shown to be upregulated in stenotic vein grafts.[6,24] PDGF-BB strongly induced HAS2 mRNA and HA synthesis in venous SMC, as has been shown before in arterial SMC[15] and mesothelial[13] cells. TGF-β1 slightly reduces HAS2 expression in mesothelial cells[13] and induces HAS2 in corneal endothelial cells.[14] However, in human venous SMC no effect of TGF-β1 on HAS2 was observed. Furthermore, PDGF-BB and TGF-β1 had no significant effects on HAS1 or HAS3.

It can be concluded that the HAS isoforms are differentially regulated by the stimuli investigated so far. HAS3 was induced only by IL-1β, HAS2 by prostaglandins, PDGF-BB, and thrombin and HAS1 was upregulated by prostaglandins only. In addition, IL-1β, PDGF-BB, and thrombin are known to induce the expression of COX-2 and the generation of endogenous prostaglandins in vascular SMC,[37,38] which might represent a mechanism to sustain HAS1 and HAS2 upregulation in SVG (Figure 8D).

Among the HAS isoforms, HAS2 was subject of the most complex regulation. Therefore, the functional significance of HAS2 induction was analyzed by siRNA targeting HAS2. Synthesis of HA and/or RHAMM signaling have been suggested to be required for G_2/M transition and cytokinesis in fibroblasts.[18,39,40] In contrast, G_1/S transition was delayed by antisense to HAS2 in epidermal keratinocytes derived from rats.[41] However, the specific role of HAS2-mediated HA synthesis during cell cycle progression in vascular SMC has not been investigated yet. In the current study, inhibition of

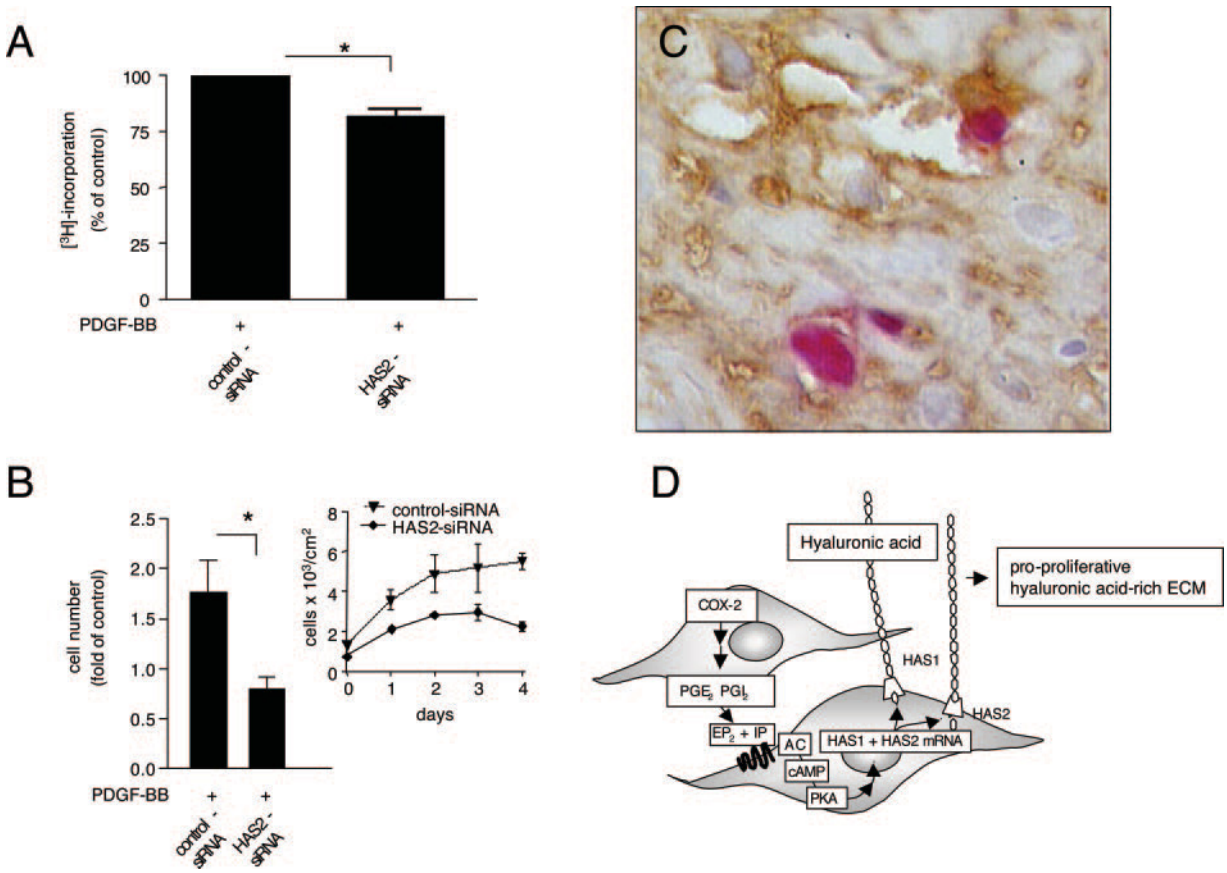

Figure 8. Inhibition of SMC proliferation by siRNA targeting HAS2. A, [^{3}H]-Thymidine incorporation 24 hours after transfection with non-silencing control siRNA and HAS2 siRNA after stimulation with PDGF-BB (20 ng/mL). Data were expressed as fold of mock transfected cells after stimulation with PDGF-BB. Statistical analysis was performed by paired *t* test. B, Cell number after stimulation of SMC that were prepared and transfected as described in A. Cell counts were performed 48 hours after stimulation by PDGF-BB. The inset shows a representative growth curve determined in triplicates of cells transfected with control siRNA and HAS2 siRNA and stimulated with PDGF-BB (20 ng/mL). C, HA and Ki-67 double staining of SMC in arterialized saphenous vein grafts. In A and B, results are mean±SEM; n=3; *$P<0.05$. D, schematic diagram illustrating that PGI_2 and PGE_2 induce HAS1 and HAS2 expression in venous SMC via stimulation of G protein–coupled IP and EP_2 receptors. These prostaglandin receptors activate adenylate cyclase (AC), which generates cAMP. Subsequent activation of PKA leads to induction of HAS1 and HAS2 expression. Increased synthesis and secretion of HA mediated by HAS2 supports SMC proliferation.

HAS2 expression caused a 20% decrease of cells in S phase, as determined by fluorescence-activated cell sorting analysis, indicating inhibition of G_1/S transition. Consistent with inhibition of G_1/S transition, the levels of cyclin A and E were dramatically reduced and the cdk inhibitor p27 was increased. Consequently, DNA synthesis was inhibited by ≈20% and cell proliferation in response to PDGF-BB was reduced as well. These findings demonstrate for the first time that HAS2-dependent HA synthesis is required for PDGF-BB–induced cell cycle progression and mitosis in vascular SMC. These data extend the observation made by others that pericellular HA coats and intracellular HA are essential for mitosis in response to PDGF-BB[15,17,18] and suggests that HAS2 mediates HA synthesis that is required for PDGF-BB induced proliferation. Proliferating SMC in venous bypass grafts were found to be frequently surrounded by HA-rich ECM, which is in line with the hypothesis that HA supports mitosis of venous SMC. Future studies will address the question whether the other HAS isoforms are involved in growth factor induced proliferation as well.

Taken together, the present study reveals that saphenous veins of elderly patients prepared for bypass grafting contain HA-rich subendothelial ECM. It is conceivable that the HA-rich ECM supports subsequent neointimal thickening because HA-rich ECM is thought to support SMC proliferation. Further evidence for this hypothesis is presented in the present study by knock down of HAS2 expression, which inhibited PDGF-BB–induced proliferation in vitro. Because the ECM of the thickened neointima of arterialized SVG is still HA rich, it is likely that the HA matrix is actively maintained via induction of HAS isoform expression in SMC. The present data show that HAS isoforms are differentially regulated by a variety of factors that are generated during pathogenesis of vein graft failure such as PDGF-BB, IL-1β, thrombin. In addition, these factors are known to induce COX-2 expression in SMC, which could subsequently cause prostaglandin release and sustained induction of HAS1 and HAS2. Therefore, endogenous prostaglandins might play a key role in the maintenance of a proproliferative HA-matrix in saphenous vein grafts.

Acknowledgments

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Arbeit #3

Rauch BH, Müschenborn B, Weber AA, Schrör K.

ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells.

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ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells

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Abstract

Fibrinogen deposition in the vessel wall represents an independent atherogenic risk factor. In Boyden-chamber assays, fibrinogen concentration-dependently (1 - 100 µM) induced migration of human vascular smooth muscle cells (SMC). This was inhibited by antibodies to intercellular adhesion molecule-1 (ICAM-1, 10 µg/ml), and by inhibitors of PI3-kinase (LY294002, 10 µM) and MAPK (mitogen-activated protein kinase) p38 (SB203580, 10 µM). The MEK (MAP kinase kinase) inhibitor PD98059 (10 µM) and the GPIIb/IIIa antagonist abciximab (10 µg/ml) had no effect. ICAM-1 antibodies inhibited fibrinogen-induced Akt and p38 phosphorylation. Thus fibrinogen stimulates human SMC migration through binding to ICAM-1 and activation of Akt and p38.

Keywords
Fibrinogen, migration, human smooth muscle cells, ICAM-1, p38 MAPK

1. Introduction

Fibrinogen is a large molecule of 340 kDa size, and consists of two sets of the polypeptide chains α, β and γ. Polymerization of fibrinogen monomers into fibrin occurs upon cleavage of fibrinopeptides A and B by thrombin (Standeven et al., 2005). Besides its role in blood clotting, fibrinogen exerts important functions in cellular interactions, inflammatory responses, wound healing, and neoplasia (Standeven et al., 2005). Fibrinogen is deposited at sites of atherosclerotic lesions in the vessel wall and hyperfibrinogenemia is considered as an independent atherogenic risk factor (Koenig, 2003).
In endothelial cells, interaction of fibrinogen with the intercellular adhesion molecule-1 (ICAM-1) mediates leukocyte adhesion and trans-endothelial invasion (Duperray et al., 1997). In smooth muscle cells (SMC), fibrinogen promotes migration (Naito et al., 1989) while its degradation products induce SMC proliferation (Naito et al., 2000). SMC also express ICAM-1 in response to inflammatory stimuli such as TNFα (Braun et al., 1995) and in pathological conditions such as atherosclerosis, restenosis and transplant vasculopathy (Braun et al., 1999). Fibrinogen-ICAM-1 interactions may therefore contribute to SMC proliferation and migration to promote vascular lesion formation. Important intracellular pathways involved in cell proliferation and migration are the PI3-kinase (PI3K)/protein kinase B (Akt) and the stress-activated mitogen-activated protein kinase (MAPK) p38 pathway, which are activated simultaneously by multiple signals and are considered key players in vascular disease (Blanc et al., 2003).
In the present study, we demonstrate for the first time that the interaction of fibrinogen with ICAM-1 induces chemotaxis in human vascular SMC through activation of Akt and p38 signaling.

2. Materials and Methods

2.1. Materials

Purified human fibrinogen type-I was obtained from Sigma-Aldrich (München, Germany). Antibodies against phosphorylated extracellular-regulated Kinase (ERK), Akt and p38 were from Cell Signaling (Frankfurt, Germany). Blocking antibodies against ICAM-1 (CD54) were from Immunotech (Marseille, France), isotype-matched control IgG was from R&D System (Wiesbaden, Germany). Abciximab was from Lilly (Bad Homburg, Germany). 2′-Amino-3′-methoxyflavone (PD98059), 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) were purchased from Calbiochem (Darmstadt, Germany). Oregon Green® 488-labeled human fibrinogen was from Molecular Probes (Eugene, USA). FITC-conjugated antibodies against ICAM-1 for flow cytometry were from Diaclone Research (Besancon, France), against α_v- (CD51) and β3-integrin (CD61) from Immunotech.

2.2. Cell Culture

Human SMC were explanted from saphenous veins obtained from patients undergoing cardiac bypass surgery. Cells were cultured as described (Rauch et al., 2002) and synchronized by serum deprivation for 48 h prior to stimulation.

2.3. Immunoblotting

Immunoblotting was performed as described (Rauch et al., 2002; Rauch et al., 2005).

2.4. DNA synthesis and cell migration

DNA synthesis was determined by [^{3}H]-thymidine incorporation as described previously (Rauch et al., 2005). Boyden chamber chemotaxis assays were performed as described (Rauch et al., 2005) with minor modifications. Cells were detached with trypsin, 20,000 cells/well were seeded to the upper chamber and allowed to migrate across a 10 µm porous polycarbonate membrane (Neuro Probe, Gaithersburg, MD, USA) for 6 h. Membranes were coated with 100 µg/ml monomeric collagen (Vitrogen, Collagen Corp., Palo Alto, CA, USA) in 0.1 M acetic acid. Cells migrated to the lower chamber were counted per high-power field.

2.5. Flow cytometry

SMC were detached non-enzymatically with citric saline buffer (0.135 M potassium chloride, 0.015 M sodium citrate) for 10 - 15 min at 37°C. Cells were pelleted, resuspended in phosphate-buffered saline, then incubated with FITC-conjugated antibodies to ICAM-1, α_v- and β3-integrin for 15 min and subjected to flow cytometry on a Beckman Coulter Epics XL 4 (Krefeld, Germany). To determine fibrinogen-binding, SMC were incubated with antibodies for 30 min prior to addition of Oregon Green® 488-labled fibrinogen for 15 min. Auto fluorescence of unlabeled cells was subtracted from mean fluorescence values of fibrinogen-labeled cells.

2.6. Statistical analysis

Data show mean±S.E.M. of n independent experiments as indicated. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. P-values of <0.05 were accepted as significant.

3. Results

3.1. Effects of fibrinogen on human SMC migration

In Boyden chamber assays, fibrinogen concentration-dependently (1 – 100 µg/ml) stimulated migration of human vascular SMC (Fig. 1A). This chemotactic effect of fibrinogen was inhibited in the presence of ICAM-1-neutralizing antibodies (10 µg/ml); an isotype-matched control IgG (mouse IgG_1, 10 µg/ml) had no effect (Fig. 1B). Fibrinogen-stimulated SMC migration was also reduced by the PI3K inhibitor LY294002 (10 µM) and by the p38 inhibitor SB203580 (10 µM), but not by the MEK (MAP kinase kinase) inhibitor PD98059 (10 µM) or by abciximab (10 µg/ml) (Fig. 1C). In addition to cell migration, a modest mitogenic effect of fibrinogen, determined by [^{3}H] thymidine incorporation, was observed at concentration of 1 – 10 µg/ml, while at 100 µg/ml fibrinogen reduced DNA synthesis (not shown).

3.2. Expression of ICAM-1 and fibrinogen-binding in human SMC

Flow cytometry demonstrated increased binding of ICAM-1 antibodies to human saphenous vein SMC, compared to isotype-matched control antibodies, indicating expression of ICAM-1 on the cell surface (Fig. 1D). Binding of fluorescently-labeled fibrinogen to SMC was inhibited in the presence of ICAM-1-neutralizing antibodies, but not by control IgG or abciximab (Fig. 1E), demonstrating a specific interaction between fibrinogen and ICAM-1 on the surface of human vascular SMC.

3.3. Fibrinogen induces phosphorylation of Akt and p38-MAPK in human SMC

Given that fibrinogen-induced SMC migration was attenuated by PI3K inhibition, which interferes with the Akt signaling pathway, and by a p38 inhibitor, we next determined whether fibrinogen causes phosphorylation of Akt and p38. Phospho-specific immunoblotting demonstrated marked phosphorylation of both kinases within 15 min exposure to fibrinogen (Fig. 2A and 2B).

3.4. Fibrinogen-induced phosphorylation of Akt and p38 depends on ICAM-1 binding in human SMC

Because ICAM-1 antibodies inhibited fibrinogen-stimulated SMC migration, we determined whether binding of fibrinogen to ICAM-1 also contributes to the observed phosphorylation of Akt and p38. In the presence of ICAM-1-neutralizing antibodies, fibrinogen-induced phosphorylation of Akt (Fig. 2C) and of p38 (Fig. 2D) was significantly inhibited. Abciximab had no effect on Akt and p38 phosphorylation. Fibrinogen-stimulated phosphorylation of ERK 1/2, seen within 15 to 30 min, was not affected by either ICAM-1 antibodies or by abciximab (not shown).

Discussion

In the present study, we investigated fibrinogen-evoked migration of human vascular SMC (Fig. 1A). A strong chemotactic response was observed at physiological concentrations of fibrinogen which range between 2 - 4 g/l (6.5 - 13 µM) (Schuitemaker et al., 2004). The purity of the used fibrinogen was confirmed by Coomassie-stained SDS-PAGE gels showing a single band for native fibrinogen and the α, β, γ chains under reducing conditions (not shown). Because fibrinogen interacts with ICAM-1 (Duperray et al., 1997), we examined whether antibodies to ICAM-1 would affect SMC migration towards fibrinogen, and found that neutralizing ICAM-1 antibodies inhibited fibrinogen-induced migration by approximately 50% (Fig. 1B and 1C). The antibody fragment abciximab (7E3) is directed against platelet GPIIb/IIIa integrin and inhibits binding of fibrinogen to activated platelets. However, abciximab has been reported to bind with similar affinity to the vitronectin receptor (αv-/β3-integrins, CD51/CD61) and to interfere with leukocyte adhesion to fibrinogen (Simon et al., 1997). Because the αv-/β3-integrin is also expressed by SMC (Jones et al., 1996), we determined whether abciximab may also affect SMC migration toward fibrinogen. However, no effect of abciximab on SMC migration was observed (Fig. 1C). We also explored possible intracellular signaling pathways which may be involved in the chemotactic effects of fibrinogen. The PI3K inhibitor LY294002 and the p38 inhibitor SB203580 were found to almost completely suppress SMC migration toward fibrinogen (Fig. 1C), suggesting involvement of signaling through Akt, which is activated by PI3K, and the stress-activated mitogen-activated kinase p38. Both kinase pathways play a role in cell migration and are often activated simultaneously (Blanc et al., 2003). Incubation of SMC with a combination of both the PI3K and the p38 inhibitors further reduced cell migration below control conditions (not shown), highlighting that both pathways are required for migration. In contrast, signaling through ERK 1/2 appears not to be essential for fibrinogen-induced SMC migration as it was not prevented by the MEK1 inhibitor PD98059 (Fig. 1C).

Increased expression of ICAM-1 has previously been reported only under inflammatory conditions such as in atherosclerotic plaque development (Braun et al.,

1995). We therefore determined the expression of ICAM-1 on the surface of human vascular SMC by flow cytometry (Fig. 1D). Because the SMC used in the present study were obtained from patients undergoing cardiac bypass surgery, inflammatory processes prior to removal of vessels may have augmented ICAM-1 expression levels in these cells. In addition, expression of α_v- (CD51) and β3-integrin (CD61) was also confirmed by flow cytometry (not shown).
Consistent with suppression of the migratory response to fibrinogen in the presence of Akt and p38 inhibitors, we found that fibrinogen increased Akt and p38 phosphorylation in a time- and concentration-dependent manner (Fig. 2A and 2B). This action was inhibited by antibodies to ICAM-1, but not by abciximab (Fig. 2D and 2C). In agreement with these observations, binding of fibrinogen to SMC was reduced by ICAM-1-neutralizing antibodies, but not by control IgG or abciximab (Fig. 1E). These data suggest that the interaction of fibrinogen with ICAM-1 on the surface of human vascular SMC results in activation of Akt and p38, while α_v- and β3-integrin appear not to be involved. In agreement with this finding, ligation of ICAM-1 has been reported to induce p38 signaling in other cell types (Lee et al., 2000). Because ICAM-1 contains only a relatively short cytoplasmatic tail, intracellular signaling though ICAM-1 may be linked predominantly to indirect effects involving rearrangement of the cytoskeleton (Cook-Mills and Deem, 2005). However, some immediate actions such as signaling through protein kinase C may also contribute (Cook-Mills and Deem, 2005). Besides the interaction with ICAM-1, binding of fibrinogen to integrins other than α_v and β_3 may also be involved, particularly since neutralizing ICAM-1 inhibited SMC migration by maximally 50% (Fig. 1B and 1C). Because fibrinogen degradation products have been implicated in SMC mitogenesis (Naito et al., 2000), we determined whether fibrinogen exerts mitogenic effects in human SMC. Interestingly, low concentrations of fibrinogen (1 – 10 µM) modestly increased DNA synthesis, but higher concentrations attenuated mitogenesis (not shown). Thus, fibrinogen appears to possess only minor mitogenic potency for human SMC. In addition to its effects on Akt and p38, fibrinogen also induced phosphorylation of ERK, which was not however affected by antibodies to ICAM-1 or by abciximab (not shown). This reflects earlier reports showing that cross-linking of ICAM-1 did not influence mitogenesis in human SMC (Lawson et al., 2001). We also determined whether fibrinogen affects cell surface expression of ICAM-1, the α_v-integrin (CD51) and the β3-integrin (CD61) by flow cytometry. However, expression levels of these molecules were not influenced by fibrinogen over 15 min to 24 h (not shown).
In summary, our data demonstrate that fibrinogen induces a strong chemotactic response in human SMC at physiological concentrations. This effect is mediated via ICAM-1-dependent activation of Akt and p38 MAPK signaling.

Acknowledgements

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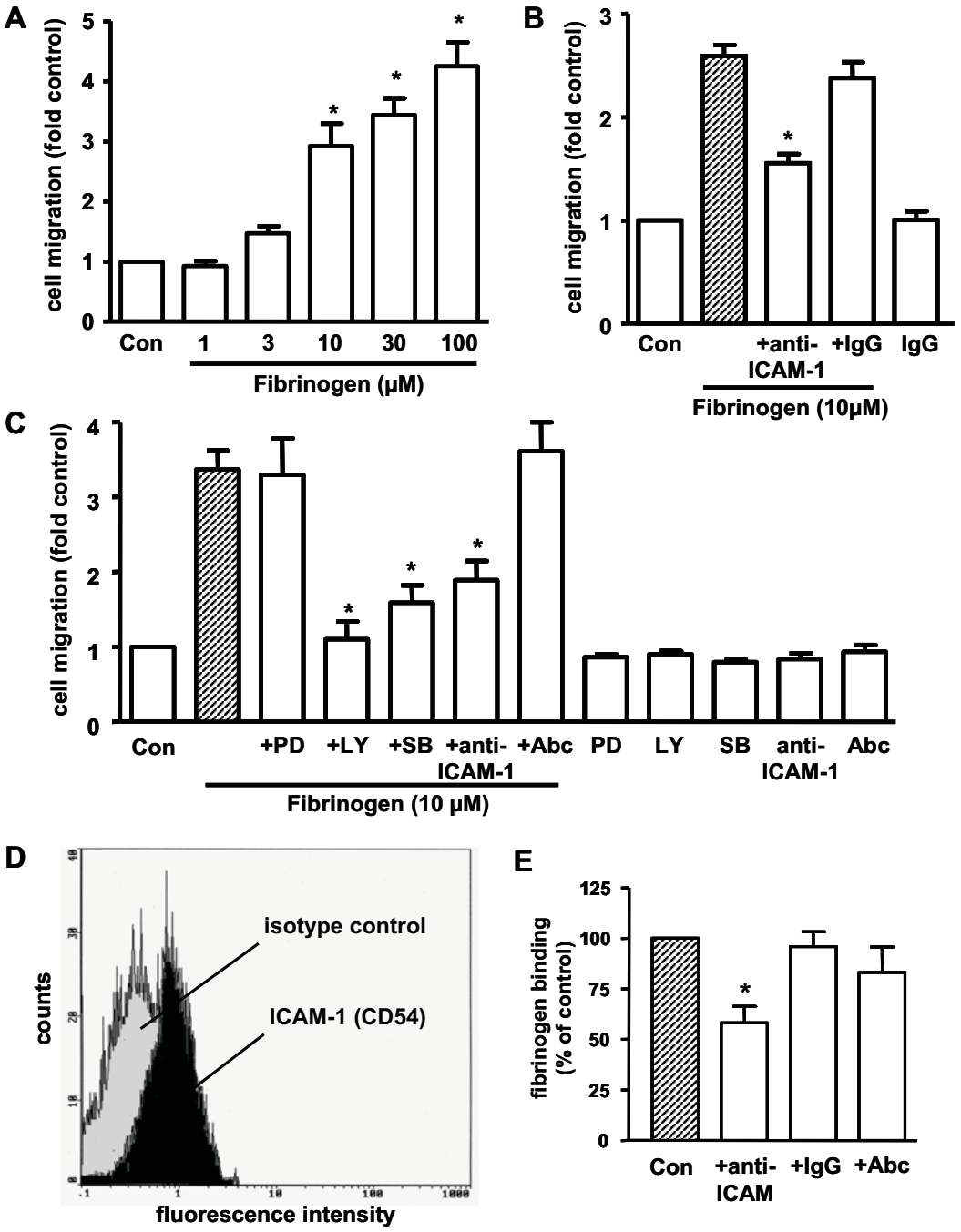

Figure 1:
Fibrinogen induces migration via binding to ICAM-1 in human SMC.
A) Human SMC were incubated with the indicated concentrations of fibrinogen in chemotaxis assays for 6 h; n=9, *P<0.05 vs. Con. **B)** Migration towards fibrinogen in absence or presence of neutralizing antibodies against ICAM-1 (anti-ICAM-1, 10 µg/ml) or a nonspecific control IgG (10 µg/ml); n=7, *P<0.05 vs. fibrinogen alone. **C)** Migration towards fibrinogen in absence or presence of MEK inhibitor PD98059 (PD, 10 µM), PI3-kinase inhibitor LY294002 (LY, 10 µM), p38 inhibitor SB203580 (SB, 10 µM), antibodies against ICAM-1 (anti-ICAM-1, 10 µg/ml), and abciximab (Abc, 10 µg/ml); n=8, *P<0.05 vs. fibrinogen alone. **D)** ICAM-1 (CD54) fluorescence on human SMC as compared to isotype control antibody, representative histogram of 6 independent experiments. **E)** Binding of fluorescence-labeled fibrinogen to human SMC in absence or presence of ICAM-1 antibodies (anti-ICAM-1, 10 µg/ml), control IgG (10 µg/ml) and abciximab (Abc, 10 µg/ml); n=4, *P<0.05 vs. fibrinogen alone.

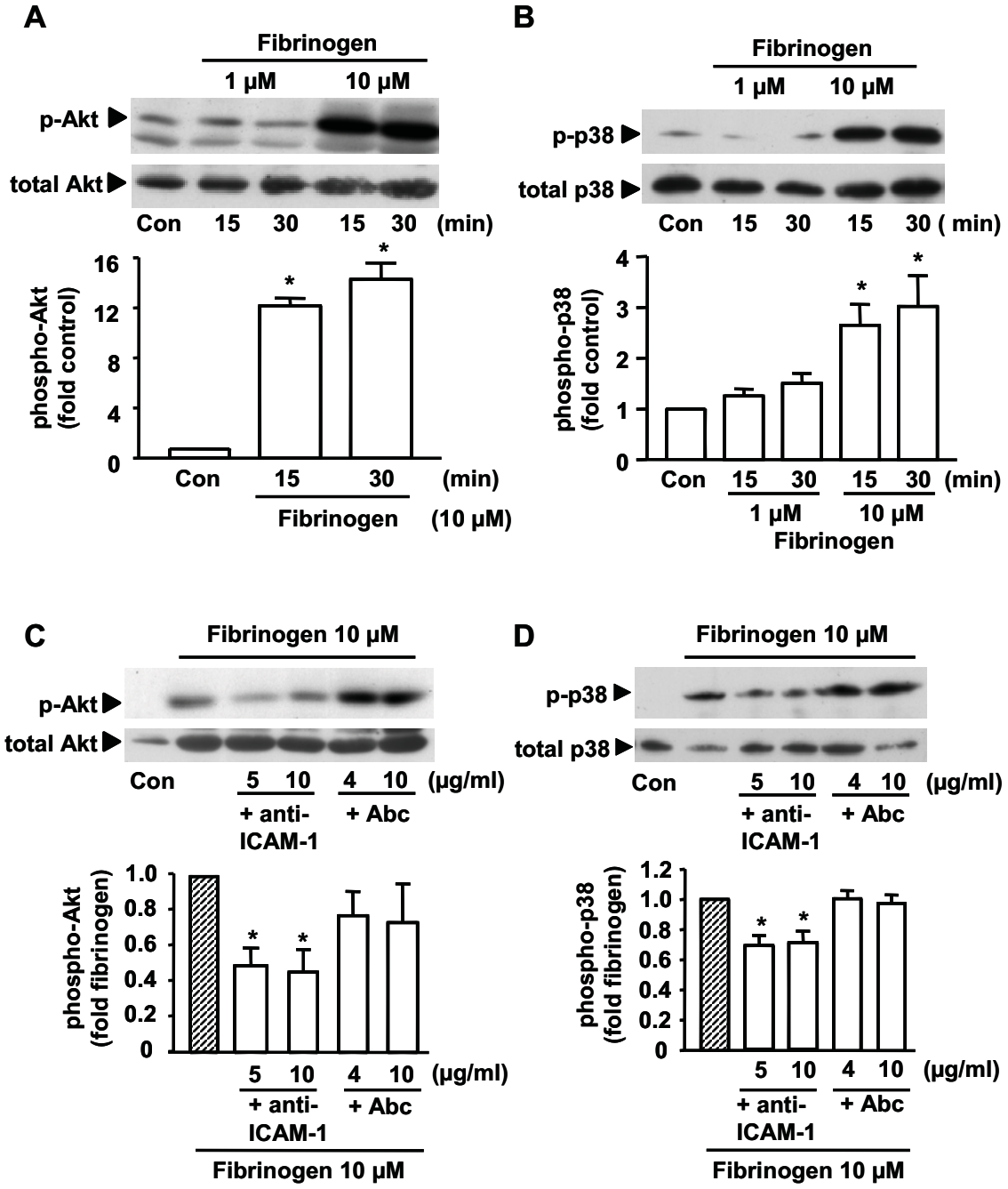

Figure 2:
Phosphorylation of Akt and p38 by fibrinogen depends on ICAM-1.
Phosphorylation of Akt **(A)** and p38 **(B)** after incubation with fibrinogen for the indicated times was determined by Western blotting. Densitometry values are expressed as fold of unstimulated controls of n=4 (A) and 5 (B) independent experiments, *P<0.05 vs. Con. Phosphorylation of Akt **(C)** and of p38 **(D)** after incubation with fibrinogen (10 µM) for 30 min in absence or presence of antibodies against ICAM-1 (anti-ICAM-1, 10 µg/ml) or abciximab (Abc, 10 µg/ml) was determined by Western blotting. Densitometry values are expressed as fold of fibrinogen-stimulated cells of n=3 (C) and 4 (D) independent experiments, *P<0.05 vs. fibrinogen alone.

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Arbeit #4

Rauch BH, Millette E, Kenagy RD, Daum G, Clowes AW.

Thrombin- and factor-Xa-induced DNA synthesis is mediated by transactivation of fibroblast growth factor receptor-1 in human vascular smooth muscle cells.

Circ Res 2004; 94:340-345.

Thrombin- and Factor Xa–Induced DNA Synthesis Is Mediated by Transactivation of Fibroblast Growth Factor Receptor-1 in Human Vascular Smooth Muscle Cells

Bernhard H. Rauch, Esther Millette, Richard D. Kenagy, Guenter Daum, Alexander W. Clowes

Abstract—Thrombin and factor Xa (FXa) are agonists for G protein–coupled receptors (GPRCs) and may contribute to vascular lesion formation by stimulating proliferation of vascular smooth muscle cells (SMCs). Mitogenic signaling of GPCRs requires transactivation of receptor tyrosine kinases (RTKs). In rat SMCs, thrombin transactivates the epidermal growth factor receptor (EGFR) via a pathway that involves heparin-binding EGF-like growth factor (HB-EGF) as ligand for EGFR. The purpose of this study was to investigate in human SMCs the role of receptor transactivation in the mitogenic response to thrombin and FXa. Thrombin (10 nmol/L) and FXa (100 nmol/L) cause a 3.3- and 2.6-fold increase in DNA synthesis, respectively. In human SMCs, neither thrombin nor FXa causes EGFR phosphorylation, and blockade of EGFR kinase does not inhibit DNA synthesis. However, DNA synthesis and phosphorylation of fibroblast growth factor receptor-1 (FGFR-1) induced by thrombin or FXa are inhibited by antibodies neutralizing basic fibroblast growth factor (bFGF) or by heparin. Hirudin inhibits thrombin-, but not FXa-induced mitogenesis, indicating that FXa acts independently of thrombin. We further demonstrate by ELISA that upon thrombin and FXa stimulation, bFGF is released and binds to the extracellular matrix. Our data suggest that in human vascular SMCs, both thrombin and FXa rapidly release bFGF into the pericellular matrix. This is followed by transactivation of the FGFR-1 and increased proliferation. Heparin may inhibit the mitogenic effects of thrombin and FXa in human SMCs by preventing bFGF binding to FGFR-1. **(*Circ Res*. 2004;94:340-345.)**

Key Words: thrombin ■ factor Xa ■ basic fibroblast growth factor ■ fibroblast growth factor receptor-1 ■ epidermal growth factor receptor

Vascular smooth muscle cell (SMC) proliferation and migration are key events in atherosclerosis and restenosis after vascular injury.[1,2] Mitogenic signaling of G protein–coupled receptors (GPCRs) involves transactivation of receptor tyrosine kinases (RTKs).[3] In several cell lines, it has been shown that the GPCR agonist thrombin mediates cell proliferation through transactivating the epidermal growth factor receptor (EGFR) via a metalloproteinase-mediated cleavage and release of pro-heparin binding EGF-like factor (HB-EGF), which then binds to EGFR.[3] We have shown that this mechanism is present in rat SMCs and is required for thrombin-induced migration.[4] Heparin, which inhibits SMC proliferation and migration in vivo and in vitro,[5–7] binds HB-EGF and interferes with this pathway.[4] We have investigated the possible role of receptor transactivation in the proliferation of human SMCs mediated by thrombin and the activated coagulation factor X (FXa). FXa is a serine protease that in addition to cleaving prothrombin activates thrombin receptors (protease-activated receptors, PARs), which are members of the GPCR family.[8] FXa acts as a thrombin-independent mitogen,[9,10] which is also sensitive to heparin inhibition.[11] In this study, we demonstrate that proliferation of human SMCs induced by thrombin and FXa does not involve EGFR transactivation, but the autocrine release of basic fibroblast growth factor and activation of the fibroblast growth factor receptor-1 (FGFR-1). This FGFR activation is inhibited by heparin and might in part account for the inhibitory effect of heparin on human SMC proliferation.

Materials and Methods

Materials

Antibodies (Abs) against FGFR-1 and EGFR were from Santa Cruz Biotechnology (Santa Cruz, Calif). The phosphotyrosine Ab (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY). EGF and the tyrphostin AG1478 were from Calbiochem (San Diego, Calif). Protein A–agarose was from Roche Diagnostics (Indianapolis, Ind). Neutralizing Ab against human basic fibroblast growth factor (bFGF) was a generous gift from Dr Michael A. Reidy (University of Washington, Seattle, Wash). Human α-thrombin was from American Diagnostica (Greenwich, Conn), and human factor Xa was from Enzyme Research Laboratories (South Bend, Ind). The bFGF ELISA kit was from R&D Systems (Minneapolis, Minn). Recombinant bFGF and heparin (porcine intestinal mucosa) were from Sigma-Aldrich (St Louis, Mo). Batimastat (BB94) was from British Biotech

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(Oxford, UK). Activating peptides (APs) for PARs were from Anaspec (San Jose, Calif): AP-1, TFFLRN (PAR-1), AP-2, SLIGKV (PAR-2), AP-3, TFRGAP (PAR-3), and AP-4, AYPGKF (PAR-4).[12]

Cell Culture

Human aortic SMCs were prepared by the explant technique as described previously.[4] Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 200 U/mL penicillin, and 200 μg/mL streptomycin. Cells at passages 5 to 12 were used for experiments.

Immunoprecipitation and Western Blotting

Immunoprecipitation (IP) of EGFR was carried out as previously described.[4] For IP of FGFR-1, cells from 100-mm culture dishes were harvested in 1 mL ice-cold HEB buffer (25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L NaF, 2 mmol/L sodium vanadate, 1 mmol/L benzamidine, 0.1% 2-mercaptoethanol, 1% Nonidet P-40, 1 mmol/L pepstatin A, 25 μg/mL leupeptin, and 20 kallikrein inhibitor units/mL aprotinin, and 1% saturated PMSF solution [in isopropanol]) and lysed for 30 minutes on ice. The lysates were cleared by centrifugation at 10 000*g* for 5 minutes in a microfuge. Three micrograms of Abs was added to the supernatants and incubated overnight at 4°C with constant agitation, then 15 μL protein A slurry was added for 1 hour at 4°C. Beads were washed once in HEB and boiled in 30 μL Laemmli sample buffer. Immunocomplexes were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed with the enhanced chemiluminescence kit (Amersham) according to the manufacturer's protocol.

DNA Synthesis

SMCs at 60% to 80% confluence were incubated for 48 hours in media without fetal bovine serum followed by a change of serum-free media for another 24 hours. Cells were stimulated by the addition of thrombin, FXa, or PAR agonistic peptides. [^{3}H]thymidine (1 μCi/mL) was added 16 to 18 hour after stimulation. After 26 to 28 hours, cells were washed 3 times with ice-cold PBS followed by incubation in 10% trichloroacetic acid (TCA) overnight at 4°C. Cells were washed in TCA, and DNA was solubilized in 0.1 N NaOH. Radioactivity was measured by liquid scintillation counting.

Enzyme-Linked Immunosorbant Assay (ELISA)

Levels of bFGF were determined using an ELISA according to the manufacturer's instructions. SMCs in 6-well plates were incubated in serum-free media for 72 hours. After stimulation, media was removed and cells were incubated with 1 mL of 10 μg/mL heparin in PBS for 20 minutes at room temperature on a rocking shaker. The heparin solution was removed, cells were detached with trypsin/EDTA, washed with PBS, and cell pellets were lysed in 1 mL HEB. Media, heparin wash, and cell lysates were stored at −80°C.

Statistics

All experiments were performed at least three times in duplicate or triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test or by a paired two-tailed *t* test as indicated. Values of $P<0.05$ were considered significant.

Results

Thrombin and FXa Do Not Transactivate EGFR in Human Vascular SMCs

In human aortic SMCs, we found no tyrosine phosphorylation of EGFR after stimulation with thrombin (10 nmol/L) or FXa (100 nmol/L) (Figure 1A). Treatment with EGF (10 ng/mL) caused a strong phosphorylation of EGFR, indicating the presence of the receptor in these cells. In addition, thrombin- and FXa-induced DNA synthesis was not affected by the specific EGFR receptor kinase inhibitor AG1478 (150 nmol/L).

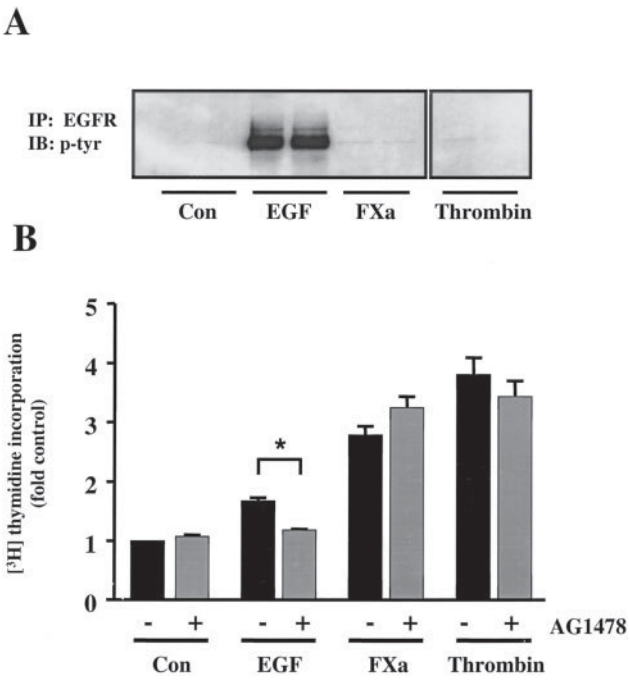

Figure 1. Thrombin and FXa do not transactivate EGFR in human SMCs. A, EGFR was immunoprecipitated from non-stimulated human SMCs (Con) or cells treated with EGF (10 ng/mL), FXa (100 nmol/L), and thrombin (10 nmol/L) for 10 minutes. Immunocomplexes (IP) were analyzed by Western blotting (IB) for phosphotyrosine (p-tyr). B, DNA synthesis was determined by [^{3}H]thymidine incorporation as described in Materials and Methods. Cells were pretreated with the EGFR kinase inhibitor AG1478 (150 nmol/L) 30 minutes before stimulation. Values are mean±SEM of 6 independent experiments. *$P<0.05$ (*t* test).

In comparison to thrombin and FXa, EGF was a weaker mitogen, and EGF-induced DNA synthesis was inhibited by AG1478 (Figure 1B). These findings suggest that EGFR is functional in human SMCs, but that thrombin and FXa do not transactivate EGFR.

Thrombin- and FXa-Induced DNA Synthesis Is Mediated by bFGF in Human Vascular SMCs

Because proliferation mediated by thrombin or FXa is inhibited by heparin, and heparin blocks FGF binding to cell surface receptors, the contribution of bFGF to thrombin- and FXa-induced DNA synthesis was investigated. SMCs were preincubated with increasing concentrations of neutralizing bFGF antibody (3 to 30 μg/mL).[13] We found a concentration-dependent inhibition of DNA synthesis stimulated by FXa or thrombin, whereas nonspecific IgG (30 μg/mL) had no effect (Figure 2). Preincubation of cells with heparin (100 μg/mL) showed an inhibitory effect comparable to bFGF neutralization (Figure 2). In contrast to thrombin-induced mitogenesis, FXa-induced DNA synthesis was not inhibited by hirudin, demonstrating that FXa acts as a thrombin-independent mitogen.

Thrombin and FXa Activate FGFR-1 in Human Vascular SMCs

FGFR-1 was immunoprecipitated from cell lysates as described in Materials and Methods. Western blotting with

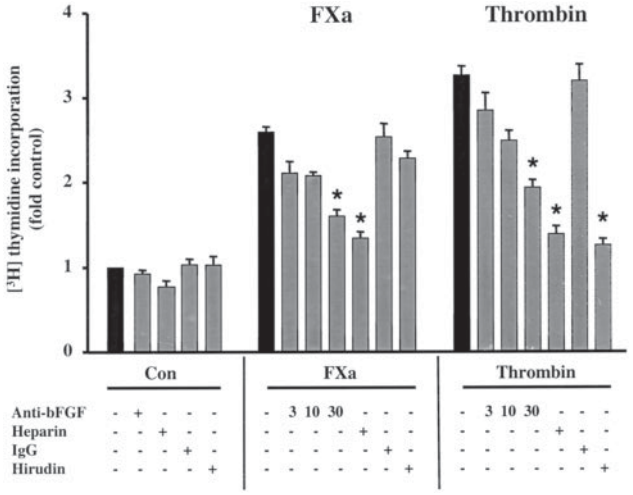

Figure 2. Thrombin- and FXa-induced DNA synthesis is mediated by bFGF. DNA synthesis in human SMCs induced by FXa (100 nmol/L) or thrombin (10 nmol/L) was determined by [^{3}H]thymidine incorporation. Cells were preincubated either with bFGF-neutralizing antibodies (3, 10, and 30 μg/mL), heparin (100 μg/mL), nonspecific IgG (30 μg/mL), or hirudin (10 μmol/L) 30 minutes before stimulation. Values are mean±SEM of 4 to 6 independent experiments. *$P<0.05$ for controls (black bars) vs indicated experimental groups (ANOVA).

anti-phosphotyrosine antibodies revealed bands of approximately 120 and 140 kDa, corresponding to FGFR-1α and β isoforms known to contain tyrosine phosphorylation sites.[14] Tyrosine phosphorylation of FGFR-1 was detected 15 minutes after stimulation with either FXa or thrombin. FGFR-1 phosphorylation was inhibited by bFGF-neutralizing antibody and heparin, but not by nonspecific IgG (Figure 3A). Thrombin- but not FXa-induced FGFR-1 phosphorylation was inhibited by preincubation with hirudin (Figure 3B). The matrix metalloproteinase (MMP) inhibitor batimastat did not prevent FGFR-1 phosphorylation by thrombin or FXa (Figure 3B). Recombinant human bFGF was used as a positive control.

Thrombin and FXa Release bFGF Into the Extracellular Matrix

Heparan sulfate proteoglycans (HSPGs) function as an extracellular matrix or cell-surface reservoir for bFGF.[15] To determine the localization of bFGF mediating the effect of thrombin and FXa, the cell layer was washed with 10 μg/mL heparin to remove any bFGF bound to the cell membrane or extracellular matrix as has been described.[16] Levels of bFGF were measured by ELISA in the media, in cell lysates, and in the heparin wash solution. Both FXa and thrombin increased bFGF in the heparin wash solution at 15 and 30 minutes (Figure 4A), whereas levels of bFGF in the media and cell lysates were not significantly different between stimulated cells and nonstimulated controls (Figures 4B and 4C, respectively). Addition of heparin before stimulation decreased bFGF bound to the cell layer by 70% (Figure 5A). At the same time, a marked increase of bFGF was measured in the cell culture media (Figure 5B).

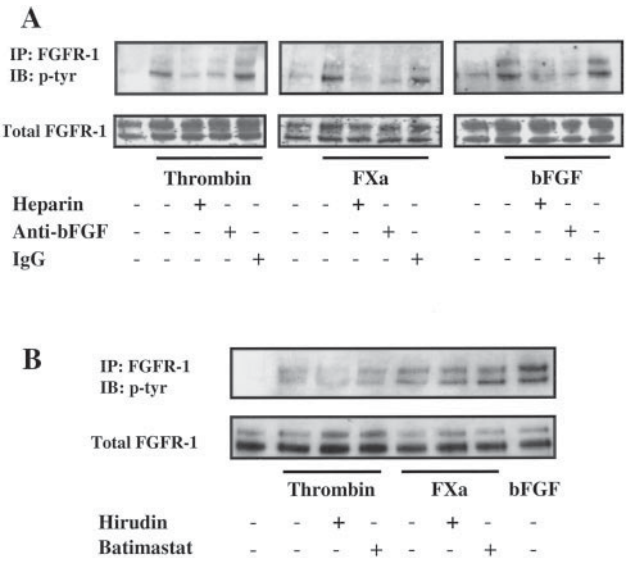

Figure 3. Thrombin and FXa transactivate FGFR-1 in human SMCs. FGFR-1 immunoprecipitates (IP) of cells stimulated with thrombin (10 nmol/L), FXa (100 nmol/L), or bFGF (20 ng/mL) for 15 minutes were analyzed by Western blotting (IB) for tyrosine phosphorylation (p-tyr). A, Cells were preincubated with bFGF-neutralizing antibodies (30 μg/mL), heparin (100 μg/mL), or nonspecific IgG (30 μg/mL) 30 minutes before stimulation. B, Cells were preincubated with hirudin (10 μmol/L) or batimastat (5 μmol/L) 30 minutes before stimulation. The double band of approximately 120 and 140 kDa presumably represents 2 different FGFR-1 isoforms.[14] Blots were reprobed for total FGFR-1.

PAR-1–Induced DNA Synthesis Is bFGF-Dependent

To determine which PARs may contribute to thrombin- and FXa-induced DNA synthesis, cells were stimulated with APs specific for PAR-1, -2, -3, and -4. Only PAR-1 agonistic peptide caused a significant increase in DNA synthesis, which was inhibited by preincubation of SMCs with either bFGF-neutralizing antibodies (30 μg/mL) or heparin (100 μg/mL) (Figure 6, data for PAR-2, -3, and -4 not shown).

Discussion

In various cell lines (HEK, COS), as well as in rat SMCs, thrombin transactivates EGFR by a metalloproteinase-dependent pathway, which involves cleavage of HB-EGF as a ligand for EGFR.[3,4,17,18] Whether EGFR transactivation in response to GPCR stimulation by thrombin plays an important role in human SMCs was the subject of this study. In contrast to rat SMCs,[4] EGFR was not transactivated by thrombin in human SMCs (Figure 1). Because thrombin signaling is inhibited by heparin in SMCs from both species, we tested the possibility that FGF is involved in human SMCs. We found that proliferation induced by thrombin and FXa is inhibited by neutralizing bFGF antibodies (Figure 2). Consistent with a role for bFGF is that both GPCR ligands release bFGF in a time-dependent manner into the pericellular matrix (Figure 4) and stimulate phosphorylation of FGFR-1 (Figure 3). In contrast to EGFR transactivation,[4] MMP inhibition by batimastat did not inhibit thrombin- or FXa-induced FGFR-1 phosphorylation (Figure 3B). The direct thrombin inhibitor hirudin reduced thrombin- but not

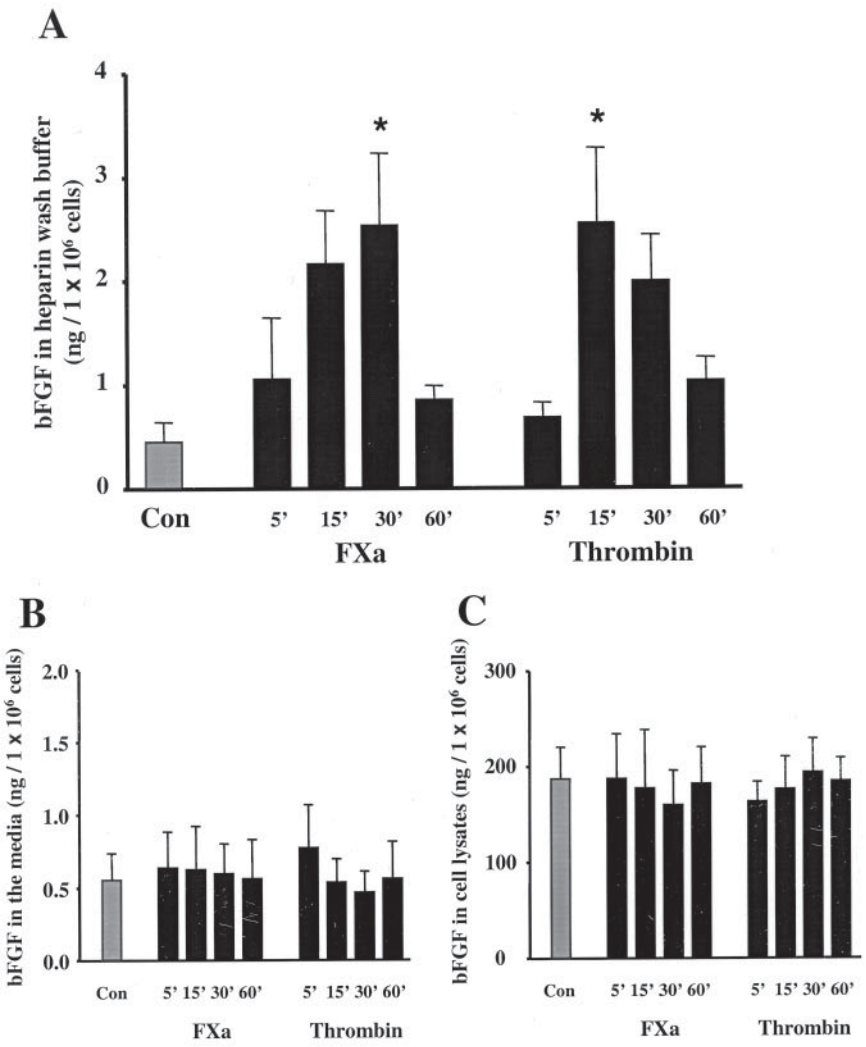

Figure 4. Thrombin and FXa release bFGF into the extracellular matrix. Basic FGF was removed from the cell layer by washing with heparin solution as described in Materials and Methods. Levels of bFGF in the heparin buffer (A), released into the media (B), and in the cells (C) after treatment with FXa or thrombin were determined by ELISA. Values are mean±SEM of 3 independent experiments. *$P<0.05$ for controls vs stimulation (ANOVA).

FXa-induced DNA synthesis (Figure 2) and FGFR-1 phosphorylation (Figure 3B), which is consistent with previous reports that FXa is a thrombin-independent mitogen.[9,10] These observations suggest that FGFR-1 mediates the mitogenic activity of thrombin and FXa, although we cannot rule out that an additional member of the FGFR family[19] is involved. Basic FGF is a mitogen for cultured SMCs and its contribution to intimal formation after arterial injury has been well described.[13,20–23] Thus, release of bFGF by GPCR ligands may be an important mechanism for the injury response. We found that bFGF is only transiently released with maximal release at 15 to 30 minutes after stimulation. It has been reported that FGFR-1 internalization peaks within 45 minutes after stimulation.[24] Thus, binding to its specific receptor and receptor internalization might contribute to the decrease of bFGF in the heparin-exchangeable bFGF fraction.

The mechanism by which bFGF is released from SMCs has not been described yet. Because bFGF lacks a typical amino acid sequence for externalization,[25] early studies speculated that bFGF is released on cell damage or by an exocytotic mechanism that is independent of the endoplasmic-reticulum-Golgi pathway.[26] Release of bFGF into the extracellular matrix is also induced by shear stress, which might cause a transient disruption of the cell membrane and thereby cause bFGF release.[16] Induction of cell membrane permeability by thrombin and the thrombin receptor (PAR-1) results in increased release of von Willebrand factor,[27] which, like bFGF, lacks an amino acid sequence for secretion. Thus, increased cell membrane permeability induced by GPCR agonists appears to be an intriguing possibility for the release of bFGF. Whether von Willebrand factor and bFGF share a common release pathway remains to be determined.

To address which PARs are involved in the mitogenic response of human SMCs, we used PAR-specific agonist peptides. We found that only the PAR-1–specific peptide caused significant DNA synthesis, suggesting that the PAR-induced mitogenic response in human aortic SMCs is mainly PAR-1–dependent. This is consistent with the ability of both thrombin and FXa to activate PAR-1.[8] Since PAR-1–induced DNA synthesis was inhibited by FGF antibodies and by heparin (Figure 6), we conclude that PAR-1 activation includes a bFGF-dependent pathway and that thrombin- and FXa-induced release of bFGF may be through PAR-1 activation. However, whether other PARs contribute to thrombin and FXa responses and to the release of bFGF and FGFR-1 transactivation requires further evaluation.

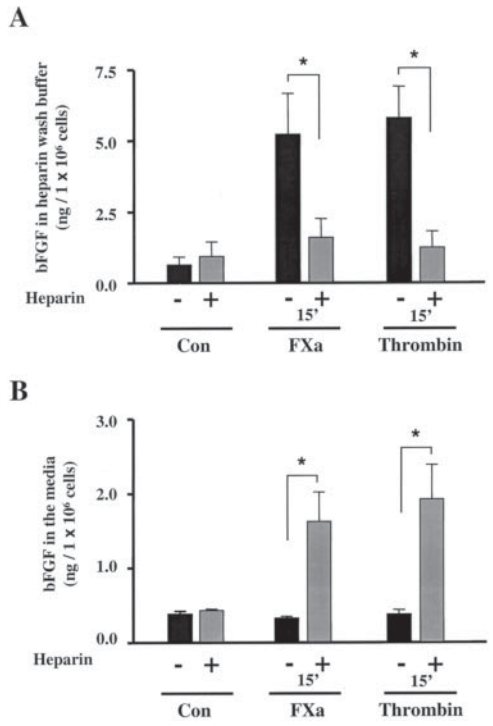

Figure 5. Pretreatment with heparin affects localization of bFGF. Cells were stimulated with FXa or thrombin for 15 minutes in the absence or presence of heparin (100 μg/mL). Basic FGF was removed from the cell layer by washing with heparin solution as described in Materials and Methods. Levels of bFGF in the heparin buffer (A) and released into the media (B) after treatment with FXa or thrombin were determined by ELISA. Values are mean±SEM of 4 independent experiments. *$P<0.05$ (*t* test).

Heparan sulfate proteoglycans (HSPGs) bind bFGF with low affinity compared with FGF receptors and are required cofactors for the activation of FGF high-affinity receptors.[15,28] Further, binding to HSPGs protects bFGF from denaturation and proteolytic degradation and provides a matrix-bound or cell-surface reservoir of this factor for the cells.[15] Heparin inhibits FGF signaling by competing with HSPGs for binding bFGF.[15,28] Our data suggest that bFGF is released from the cells after stimulation with thrombin and FXa and binds to HSPGs in the pericellular matrix (Figures 4A and 5). However, when high concentrations of heparin are present, binding of bFGF to the matrix is inhibited, indicated by the presence of bFGF in the heparin-containing media (Figure 5). One example of a bFGF binding HSPG is syndecan-4, which is a cofactor for FGFR-1 activation in fibroblasts.[29] Recently, it has been reported that bFGF and syndecan-4 may also be involved in the regulation of integrin functions,[30,31] which might be involved in a bFGF-dependent thrombin- and FXa-induced mitogenic response.

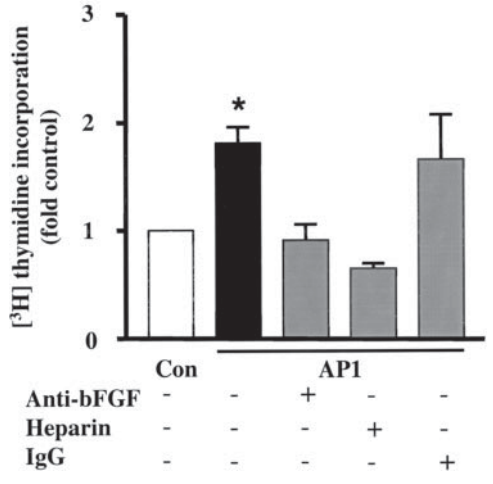

Figure 6. PAR-1–induced DNA synthesis is bFGF-dependent. Cells were stimulated with activating peptide (AP1, 100 μmol/L) specific for PAR-1. DNA synthesis was determined by [^{3}H]thymidine incorporation. Cells were preincubated either with bFGF-neutralizing antibodies (30 μg/mL), heparin (100 μg/mL), or non-specific IgG (30 μg/mL) 30 minutes before stimulation with AP-1 (100 μmol/L). Values are mean±SEM of 3 independent experiments. *$P<0.05$ (ANOVA).

In summary, our data indicate that in human SMCs PAR-1 signaling involves activation of FGFR-1. This is different from recent observations in rat SMCs, where thrombin transactivates EGFR. Our study demonstrates that thrombin and FXa cause autocrine FGFR-1 phosphorylation via release of bFGF in human SMCs. This mechanism might contribute to thrombin- and FXa-induced mitogenesis. Our data suggest further that heparin inhibits mitogenesis induced by these stimuli by competing with HSPGs for released bFGF, which prevents FGFR-1 phosphorylation.

Acknowledgments

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Arbeit #5

Millette E, Rauch BH, Kenagy RD, Daum G, Clowes AW.

Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation.

Circ Res 2005; 96: 172-179.

Platelet-Derived Growth Factor-BB–Induced Human Smooth Muscle Cell Proliferation Depends on Basic FGF Release and FGFR-1 Activation

Esther Millette, Bernhard H. Rauch, Olivier Defawe, Richard D. Kenagy, Guenter Daum, Alexander W. Clowes

Abstract*—**We have shown that the G protein–coupled receptor (GPCR) agonists, thrombin and Factor Xa, stimulate smooth muscle cell (SMC) proliferation through transactivation of the EGF receptor (EGFR) or the FGF receptor (FGFR), both of which are tyrosine kinase receptors. In the present study, we investigated whether platelet-derived growth factor (PDGF), a tyrosine kinase receptor agonist, might transactivate another tyrosine kinase receptor to induce SMC proliferation. Because heparin inhibits PDGF-mediated proliferation in human SMCs, we investigated whether the heparin-binding growth factor basic fibroblast growth factor (bFGF) and one of its receptors, FGFR-1, play a role in the response of human arterial SMCs to PDGF-BB. PDGF-BB induced the release of bFGF and sustained phosphorylation of FGFR-1 (30 minutes to 6 hours). A bFGF-neutralizing antibody inhibited PDGF-BB–mediated phosphorylation of FGFR-1, DNA synthesis, and cell proliferation. In the presence of bFGF antibody, PDGF-BB–induced early activation of ERK (0 to 60 minutes) was not affected, whereas late ERK activation (2 to 4 hours) was reduced. When FGFR-1 expression was suppressed using small interfering RNA (siRNA), ERK activation was reduced at late, but not early, time points after PDGF-BB stimulation. Addition of bFGF antibody to cells treated with siRNA to FGFR-1 had no further effect on ERK activation. Our results provide support for a novel mechanism by which PDGF-BB induces the release of bFGF and activation of FGFR-1 followed by the sustained activation of ERK and proliferation of human SMCs. (Circ Res*. 2005;96:172-179.)**

Key Words: platelet-derived growth factor ■ bFGF release ■ FGFR-1 ■ ERK ■ Akt

Activation of the ERK pathway is critical for cell proliferation.[1] Although mitogens appear to activate maximally ERK within 15 minutes, prolonged activity of ERK is necessary for cell cycle progression through G_1/S.[2–4] Several authors have described two waves of signaling in response to mitogens: the first leads cells into G_1, whereas the second is responsible for progression from G_1 to S phase.[3,5,6] For mitogens that bind to G protein–coupled receptors (GPCRs), the second wave of signaling depends on a receptor-linked tyrosine kinase.[3,7,8] For example, in rat smooth muscle cells (SMCs), sustained ERK activity in response to thrombin and angiotensin II involves the release of heparin-binding EGF-like growth factor (HB-EGF), which in turn, activates the EGF receptor (EGFR).[7,9,10] Heparin may inhibit thrombin-induced migration and proliferation of rat SMCs, in part, by preventing the binding of HB-EGF to heparan sulfate proteoglycans (HSPGs),[7,11] because heparin binding growth factors (eg, HB-EGF, basic fibroblast growth factor [bFGF]) require HSPGs as coreceptors for biological activity.[12]

Like GPCR ligands, platelet-derived growth factor (PDGF)-BB induces two waves of signaling, and the second peak of ERK and phosphatidylinositol 3 (PI3)-kinase activity is required for progression through G_1.[6] However, EGFR transactivation is not involved.[7] Of significant interest is the observation that ERK activity induced by PDGF-BB is inhibited at late time points by heparin.[13] This suggests that heparin binding growth factors, other than EGFR ligands, may be involved in the response to PDGF-BB. In this regard, we recently found that thrombin- and factor Xa–induced proliferation of human SMCs depends on endogenous bFGF.[14] In addition, lysophosphatidylcholine was reported to induce migration and angiotensin II to induce proliferation of human SMCs through the release of bFGF.[15,16] For these reasons, we hypothesized that endogenous bFGF mediates the proliferative activity of PDGF-BB, a tyrosine kinase receptor agonist. In this study, we report a novel role for endogenous bFGF and FGF receptor (FGFR)-1 in PDGF-BB–mediated human SMC proliferation. In addition, prolonged activity of ERK is required for cell cycle progression and is dependent on bFGF-FGFR-1.

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Materials and Methods

Materials

PDGF-AA and -BB were a gift from Zymogenetics (Seattle, Wash). PDGF-CC, PDGF-DD, and the bFGF ELISA kit were purchased from R&D Systems. Anti–smooth muscle-α-actin, recombinant bFGF, actinomycin D, and heparin (porcine intestinal mucosa) were from Sigma-Aldrich. AG1296, PD98059, LY294002, and GF109203X (bisindolylmaleimide) were from Calbiochem. Neutralizing Ab against human basic fibroblast growth factor (bFGF) and ERK1/2 antiserum were generous gifts from Drs Michael Reidy[17] and Karen Bornfeld (University of Washington, Seattle, Wash), respectively. The phosphotyrosine Ab (clone 4G10) was from Upstate Biotechnology, and the Ab against FGF receptor-1 was from Santa Cruz Biotechnology. Ab against phosphorylated ERK1/2 and Akt were obtained from Cell Signaling Technology. Ab against phosphorylated MEK 1/2 was obtained from New England Biolabs. Protein A-agarose was from Roche Diagnostics. All cell culture solutions were from Gibco.

Cell Culture

Human SMCs were prepared from abdominal aortas of organ donors with the approval of the Human Subject Review Board of the University of Washington. After removing endothelial cells, the inner part of the media was dissected and minced into explants, which were maintained in DMEM with 10% serum. After passage, the cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 200 U/mL penicillin, and 200 μg/mL streptomycin. Cell lines were used from passage 5 to 12.

Basic FGF Immunoassay

SMCs in 6-well plates were serum-deprived for 72 hours. At different times after stimulation, media was removed and cells were incubated with heparin (10 μg/mL) in PBS for 30 minutes at room temperature on a shaker. The heparin solution was removed and cells were detached with trypsin/EDTA. Cells were pelleted in a microcentrifuge and lysed in 1 mL HEB (25 mmol/L HEPES-NaOH, pH 7.5, 1 mmol/L EDTA, 150 mmol/L NaCl, 10 mmol/L NaF, 2 mmol/L sodium vanadate, 1 mmol/L benzamidine, 1% Nonidet P-40, 0.1% 2-mercaptoethanol, 1 mmol/L pepstatin A, 2 μg/mL leupeptin, and 20 kallikrein inhibitor units/mL aprotinin) and 20 μmol/L of phenylmethylsulfonyl fluoride. Levels of bFGF in media, heparin-wash, and cell lysates were determined by ELISA according to the manufacturer's instructions.

^{3}H-Thymidine Incorporation

Cells at 60% to 80% confluence were serum-deprived for 48 to 72 hours. Cells were stimulated with PDGF (10 ng/mL) and [^{3}H]-thymidine (1 μCi/mL) was added 18 hours later. After another 8 hours, [^{3}H]-thymidine incorporation was determined as previously described.[14]

Migration Assay

Microchemotaxis assays were performed as described[18] for 5 hours at 37°C with 48-well chambers (Neuro Probe) and polycarbonate filters (10-μm pores; Nucleopore Corp) coated with monomeric collagen (100 μg/mL Vitrogen 100 in 0.1 mol/L acetic acid; Collagen Corp). Cells (35 000/well) were added to the upper chamber and chemoattractants or DMEM were added to the lower chamber. The migrating cells per high-power field were counted.

Growth Curve

SMCs were seeded (1200 cells/cm^2) with media containing 10% FBS. The following day, media was changed to 0.5% FBS, and cells were stimulated 4 hours later. The media was changed every 48 hours. Cells were counted by Coulter counter (Beckman).

Immunoprecipitation of FGFR-1 and Western Blot Analysis

Immunoprecipitation of FGFR-1 was performed as previously described.[14] Samples were subjected to SDS-PAGE (7% for FGFR-1 and 10% for ERK, Akt, MEK) and transferred to nitrocellulose membranes. Membranes were probed with antibodies at 4°C overnight. Immunodetection was performed by enhanced chemiluminescence (Amersham). Densitometric analysis of bands was performed using Image Quant (Molecular Dynamics).

Preparation of Small Interfering RNAs Targeting FGFR-1

Small interfering RNAs (siRNAs) targeting human FGFR-1 were designed using a siRNA construction kit, (Ambion) and were constructed from sense and anti-sense DNA oligonucleotides (Integrated DNA Technologies) using Basic Local Alignment Search Tool (BLAST) to avoid homology with other mRNA. The sense sequence for FGFR-1 siRNA was 5′-AAGTCGGACGCAACAGAGAAA-3′. The sequence for the scrambled siRNA (control) was 5′-AACAGAGAAAGTCGGACGCAA-3′.

Cells were transfected in 100-mm dishes with siRNAs (10 nmol/L) by calcium phosphate-precipitation for 15 hours, as described.[19] Cells were washed three times with PBS, twice with media containing 15% FBS, and allowed to recover for at least 9 hours. For DNA synthesis and Western blot analysis for phosphoERK, cells were trypsinized and seeded into 12-well plates.

Statistical Analysis

All experiments were repeated at least four times. Statistical analyses were performed using one-way ANOVA followed by Bonferroni multiple comparison test or by a paired two-tailed *t* test as indicated. Statistical significance was accepted at $P \leq 0.05$.

Results

Endogenous bFGF Contributes to PDGF-BB–Induced Cell Proliferation, but Not Migration

In human SMCs, we found a strong inhibitory effect of heparin (100 μg/mL) on PDGF (AA, BB, CC, or DD)-induced DNA synthesis (Figure 1A). To determine whether an HSPG-sensitive EGFR ligand was involved, we blocked EGFR kinase activity with the selective inhibitor AG1478 (150 nmol/L). Because this treatment did not reduce PDGF-BB–induced DNA synthesis (unpublished data, 2002), we tested the involvement of bFGF. Preincubation of SMCs with bFGF antibody (30 μg/mL) inhibited DNA synthesis induced by the PDGF ligands (Figure 1A). In addition, cell proliferation induced by PDGF-BB was inhibited by 90±15% by the bFGF antibody and was completely abolished by heparin (Figure 1B).

In contrast to proliferation, the bFGF antibody did not inhibit PDGF-BB-induced migration of human SMCs (Figure 1C). In control experiments, bFGF antibody inhibited migration in response to bFGF (Figure 1C).

PDGF-BB Activates FGFR-1

To investigate whether PDGF-BB activates FGFR-1, cells were stimulated with PDGF-BB for 30 minutes to 6 hours. Phosphorylation of FGFR-1 was increased within 30 minutes, lasted at least 6 hours, was comparable to receptor phosphorylation caused by bFGF (Figure 2A) and was inhibited by the bFGF antibody and heparin (Figure 2B).

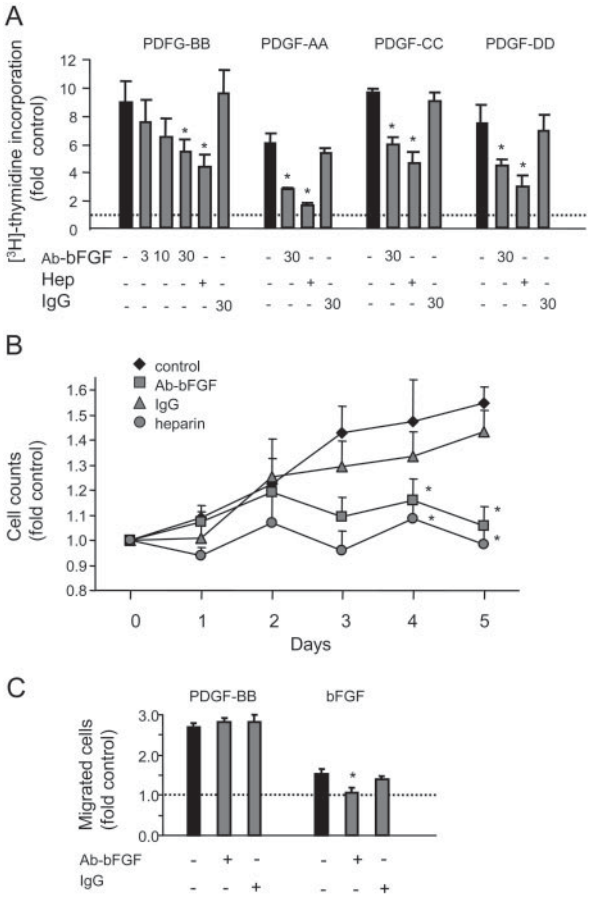

Figure 1. Basic FGF contributes to SMC growth, but not migration, in response to PDGF-BB. A, Effect of bFGF antibody (3, 10, and 30 μg/mL), heparin (HEP, 100 μg/mL), or nonspecific IgG (30 μg/mL) on DNA synthesis induced by PDGF-AA, -BB, -CC, and -DD (10 ng/mL) in human SMCs. Cells were preincubated with factors 30 minutes before PDGF stimulation. Data (mean±SEM of 5 independent experiments) are presented as fold increase over nonstimulated cells (dotted line). B, Cell proliferation induced by PDGF-BB was determined by cell counts from day 1 to day 5. Cells were preincubated without (black diamond) or with bFGF antibody (gray square, 30 μg/mL), heparin (gray circle, 100 μg/mL), or nonspecific IgG (gray triangle, 30 μg/mL) 30 minutes before stimulation with PDGF-BB. Data are presented as in A (n=6). C, Effect of preincubation for 1 hour with either bFGF antibody (30 μg/mL) or nonspecific IgG (30 μg/mL) on migration of SMCs toward PDGF-BB (10 ng/mL) or bFGF (10 ng/mL). Data are presented as in A (n=5). *$P\leq0.05$ vs control stimulation (black).

PDGF-BB Releases bFGF

The effect of the bFGF antibody suggests that bFGF was released by SMCs in response to PDGF. To test this hypothesis, levels of bFGF in the media, in the cell/matrix layer (released by heparin), and in intracellular stores were measured. After stimulation with PDGF-BB, bFGF increased slightly in the culture media at 1 hour (Table). In the cell/matrix layer, the levels of bFGF increased by 2-fold within 30 minutes and 8-fold after 4 hours (Figure 3). Basic FGF in the cell/matrix layer remained elevated at 24 hours (Figure 3). In contrast, there was no difference in the levels of intracellular bFGF between PDGF-BB–stimulated cells and unstimulated control cells (Table).

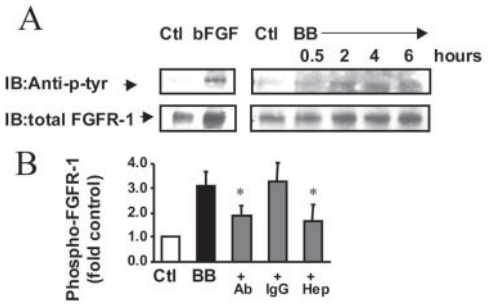

Figure 2. Phosphorylation of FGFR-1 in response to PDGF-BB. A, Quiescent SMCs (Ctl) were stimulated for 15 minutes with bFGF (1 ng/mL) or for 30 minutes to 6 hours with PDGF-BB (BB, 10 ng/mL). Cells were lysed, FGFR-1 was immunoprecipitated, and immunocomplexes were analyzed by Western blotting for phosphotyrosine (Anti-p-tyr) and FGFR-1 protein. Representative blot from 3 independent experiments with similar results is shown. B, Quiescent SMCs (Ctl) were stimulated for 4 hours with PDGF-BB (BB, 10 ng/mL). Where indicated, bFGF antibody (Ab, 30 μg/mL), nonspecific IgG (30 μg/mL), or heparin (Hep, 100 μg/mL) was added 30 minutes before PDGF-BB stimulation. Data (mean±SEM of 5 independent experiments) are presented as fold intensity over nonstimulated samples. *$P\leq0.05$, PDGF-BB with anti-bFGF vs PDGF-BB with IgG; PDGF-BB with heparin vs control PDGF-BB (black).

Time Window of Stimulatory Effect of bFGF

To determine when bFGF is required for PDGF-BB–induced DNA synthesis, cells were incubated with bFGF antibody 30 minutes before or 2 to 16 hours after PDGF-BB stimulation. As shown in Figure 4, treatment of cells with bFGF antibody could be delayed up to 4 hours without significantly reducing the inhibitory effect, whereas further delay resulted in a loss of inhibition. This suggests that the stimulatory effect of bFGF on PDGF-BB–induced DNA synthesis occurs within the first 4 to 6 hours.

Basic FGF Contributes to PDGF-BB–Induced MEK and ERK Activation

To determine whether the activation of the ERK signal transduction pathway by PDGF-BB depends on endogenous bFGF, quiescent cells were stimulated with PDGF-BB, in the absence or the presence of bFGF antibody, nonspecific IgG, or heparin. PDGF-BB induced two peaks of ERK activation: the first was observed within 15 to 30 minutes, whereas the second was observed between 2 to 4 hours (Figure 5A and 5B). Preincubation of SMCs with bFGF antibody or heparin

Basic FGF Levels (ng/10^6 cells) in the Culture Media, the Matrix Plus Cell Surface, or Intracellular Pool After PDGF-BB Stimulation

	Media	Pericellular	Intracellular
No stimulation	0.41±0.10	1.05±0.39	189.56±18.46
30 minutes	0.44±0.19	2.21±0.50*	200.44±37.18
1 hour	0.84±0.09*	2.25±0.32*	174.01±37.20
4 hours	0.77±0.16	8.36±1.24*	174.62±19.68

SMCs were stimulated with PDGF-BB (10 ng/ml) for the indicated times. Nonstimulated control was included. As described in Materials and Methods, levels of bFGF in the media, in the pericellular layer (released by heparin), and in intracellular stores were measured by ELISA. Data are the mean±SEM of 4 to 7 independent experiments. *$P=0.05$ vs no stimulation.

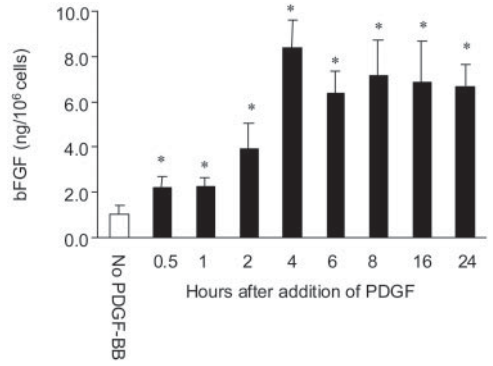

Figure 3. Basic FGF accumulates in extracellular matrix and the cell surface after stimulation with PDGF-BB. SMCs were stimulated with PDGF-BB (10 ng/mL) for the indicated times (black bars). Nonstimulated control was included (white bar). Basic FGF in the heparin-releasable fraction was determined by ELISA. Data are the mean±SEM of 4 to 7 independent experiments. *$P \leq 0.05$, vs no stimulation (white bar).

did not affect early ERK activation (0 to 60 minutes), but inhibited PDGF-BB–induced ERK activation at 2 and 4 hours ($P<0.05$) and at 8 hours ($P=0.06$) (Figure 5A and 5B). Consistent with this observation, PDGF-BB–induced phosphorylation of the ERK activator, MEK 1/2, was not altered by bFGF antibody or heparin at 15 to 30 minutes, but was inhibited by both after 2 and 4 hours (Figure 5A).

Because PI3-kinase is known to contribute to PDGF-BB–induced mitogenesis, we investigated whether bFGF is required for the activation of Akt, a downstream protein kinase in the PI3-kinase signaling pathway. Preincubation of SMCs with bFGF antibody or heparin did not affect phosphorylation of Akt in response to PDGF-BB (Figure 5A), suggesting a specific effect of bFGF on MEK/ERK activity.

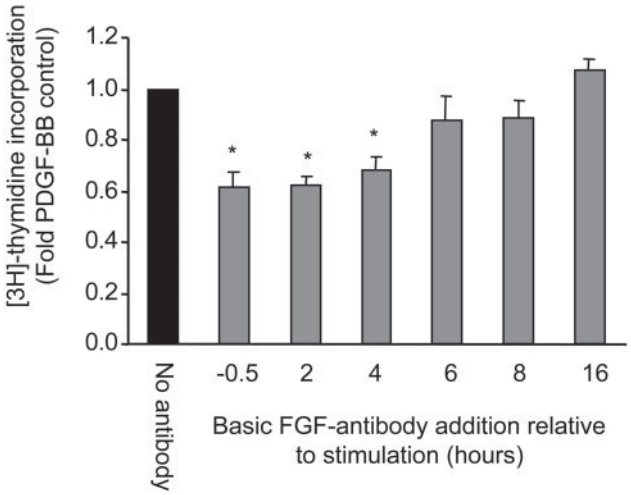

Figure 4. Time window of the stimulatory effect of bFGF. Basic FGF-antibody was added (gray bars) at the indicated time points after stimulation with 10 ng/mL of PDGF-BB. PDGF-BB stimulation without antibody is represented by the black bar. DNA synthesis was determined by [^{3}H]-thymidine incorporation. Data are the mean±SEM of 5 independent experiments. *$P \leq 0.05$ vs PDGF-BB alone (black bar).

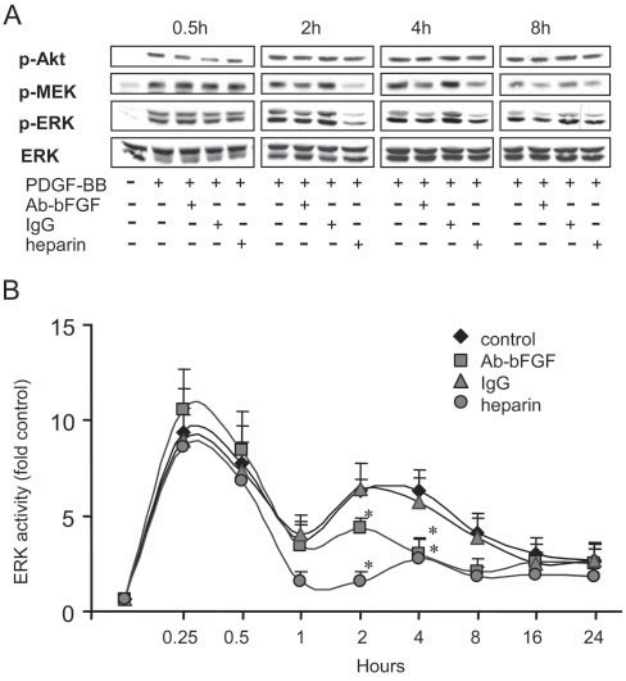

Figure 5. Basic FGF contributes to PDGF-BB–induced late ERK activation. Quiescent cells were stimulated with PDGF-BB (10 ng/mL) for various times (30 minutes to 24 hours), as indicated in the absence (black diamond) or presence of bFGF antibody (gray square, 30 μg/mL), nonspecific IgG (gray triangle, 30 μg/mL), or heparin (gray circle, 100 μg/mL). A, Representative blot for phosphorylation of Akt (p-Akt), MEK (p-MEK), and ERK (p-ERK) of 5 independent experiments is shown. B, Densitometric analysis of phosphoERK. Data (mean±SEM; n=5) are presented as fold intensity over nonstimulated samples. *$P \leq 0.05$ vs control PDGF-BB stimulation (black).

FGFR-1 Contributes to PDGF-BB–Induced ERK Activation and DNA Synthesis

FGFR-1 is reported to be the main receptor contributing to bFGF-induced mitogenesis in rat SMCs.[20] To investigate the role of FGFR-1 in PDGF-BB–induced mitogenesis in human SMCs, we used siRNA to downregulate FGFR-1. As shown in Figure 6A, treatment of cells with FGFR-1 siRNA downregulated FGFR-1 expression from day 2 to 4 after transfection, while not affecting levels of smooth muscle-α-actin. In addition, there was no difference in cell number between cells transfected with FGFR-1 or scrambled siRNA (6978±623 and 7079±665 cells at day 4, respectively, n=4). Downregulation of FGFR-1 inhibited DNA synthesis in response to PDGF-BB (Figure 6B) and bFGF antibody did not further reduce PDGF-BB–induced [^{3}H]-thymidine incorporation in FGFR-1 downregulated cells. In contrast, heparin was still inhibitory, suggesting additional mechanisms for heparin-mediated inhibition of PDGF-BB–induced DNA synthesis.

To confirm a role for FGFR-1 in PDGF-BB signaling, we investigated whether FGFR-1 downregulation also affects late ERK phosphorylation (Figure 6C). Although bFGF-mediated ERK activation at 30 minutes was completely blocked in FGFR-1 downregulated cells, PDGF-BB–induced ERK activation was similar in FGFR-1 and scrambled siRNA-treated cells. In contrast, at 4 hours PDGF-BB–mediated ERK activation was reduced in FGFR-1 downregu-

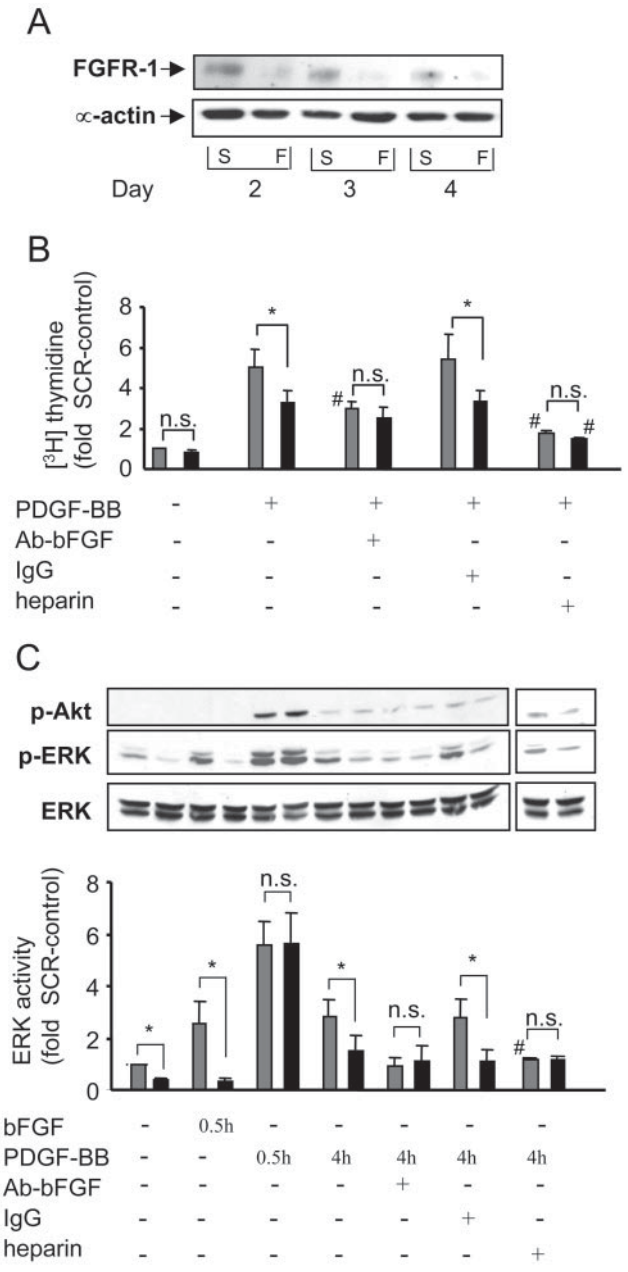

Figure 6. A, Downregulation of FGFR-1 by siRNA. FGFR-1 was immunoprecipitated from cell lysates prepared on days 2, 3, and 4 after transfection with siRNA against FGFR-1 (F) or scrambled siRNA (S). Representative Western blot for FGFR-1 of 4 independent experiments is shown. To ensure equal amounts of proteins, an aliquot of the cell lysates was analyzed by Western blotting for expression of smooth muscle-α-actin (α-actin). B, Downregulation of FGFR-1 inhibits PDGF-BB–induced [^{3}H]-thymidine incorporation into DNA. On day 0, cells were transfected with scrambled siRNA (gray bars) or siRNA targeting FGFR-1 (black bars). On day 4, quiescent SMCs were stimulated with PDGF-BB (10 ng/mL). Where indicated, cells were incubated with bFGF antibody (30 μg/mL), nonspecific IgG (30 μg/mL), or heparin (100 μg/mL) 30 minutes before stimulation. Data (mean±SEM of 5 independent experiments) are presented as fold stimulation over unstimulated, scrambled siRNA cells. C, Downregulation of FGFR-1 inhibits PDGF-BB–induced late ERK activation. SMCs were transfected as described in B, Quiescent cells were stimulated with bFGF (1 ng/mL) for 30 minutes or with PDGF-BB (10 ng/mL) for 30 minutes or 4 hours. Where indicated, cells were incubated with bFGF antibody (30 μg/mL), nonspecific IgG (30 μg/mL), or heparin (100 μg/mL) 30 minutes before PDGF-BB stimulation. Representative blot for p-Akt, p-ERK, and ERK of 4 independent experiments is shown. Data (mean±SEM) for p-ERK (bottom panel) are presented as fold stimulation over unstimulated, scrambled siRNA cells. #$P \leq 0.05$ vs control 4-hour PDGF-BB stimulation; *$P \leq 0.05$, FGFR-1 siRNA vs scrambled siRNA-treated cells; *t* test analysis.

lated cells. In addition, bFGF antibody and heparin had no further inhibitory effect on ERK phosphorylation at 4 hours in FGFR-1 downregulated cells (Figure 6C). Depletion of FGFR-1 did not affect Akt phosphorylation by PDGF-BB (Figure 6C).

We investigated whether downregulation of FGFR-1 affects PDGF-BB–induced migration in human SMCs. Treatment of cells with FGFR-1 siRNA did not alter PDGF-BB–induced migration (unpublished data, 2004). This is consistent with the lack of an inhibitory effect of the bFGF antibody on PDGF-BB–induced migration in control cells (Figure 1C).

Signaling Proteins Involved in PDGF-BB–Induced bFGF Release

To investigate the pathways signaling in PDGF-BB–mediated bFGF release, quiescent cells were stimulated for 4 hours with PDGF-BB, in the absence or presence of 20 μmol/L AG1296 (AG, an inhibitor of PDGF receptor kinase), 10 μmol/L LY294002 (LY, a PI3-kinase inhibitor), 40 μmol/L PD98059 (PD, a MAPK inhibitor), or 10 μmol/L GF109203X (GFX, a PKC inhibitor). After 4 hours of stimulation with PDGF-BB, bFGF increased in the pericellular layer (heparin releasable; Figure 7A). Pretreatment of the cells with AG, LY, or GFX, but not PD, reduced PDGF-BB–induced bFGF levels in the pericellular layer (Figure 7A), suggesting that PI-3 kinase and PKC are involved in PDGF-BB–induced bFGF release and that PDGF receptors mediate this effect.

To determine the effect of these inhibitors on phosphorylation of FGFR-1, cells were stimulated with PDGF-BB for 4 hours in the absence or the presence of AG, PD, LY, or GFX. Consistent with the previous data showing an inhibitory effect of AG, LY, and GFX on bFGF release, all but PD reduced PDGF-BB–induced FGFR-1 phosphorylation (Figure 7B).

Finally, we investigated the role of PDGF receptor kinase, PKC, and PI3-kinase on the sustained, bFGF-dependent, activity of ERK. Preincubation of SMCs with AG, LY, and GFX reduced the phosphorylation of ERK 4 hours after PDGF-BB stimulation (Figure 7C). Thus, PDGF-BB–induced bFGF release, FGFR-1 phosphorylation, and sustained ERK activity depend on PDGF receptor kinase, PKC and Akt activation. In addition, PDGF-BB–induced Akt activity, which is not affected by bFGF antibody (Figure 5), was unaltered by the MAPK inhibitor (Figure 7C), but was abolished by the PDGF receptor kinase inhibitor (Figure 7C), confirming that PDGF-BB activates Akt independently of bFGF and sustained MAPK activity.

We investigated whether bFGF transcription is required for PDGF-BB to induce bFGF release. We found that release of bFGF after 4 hours of stimulation with PDGF-BB was not altered by actinomycin D starting 1 hour before treatment

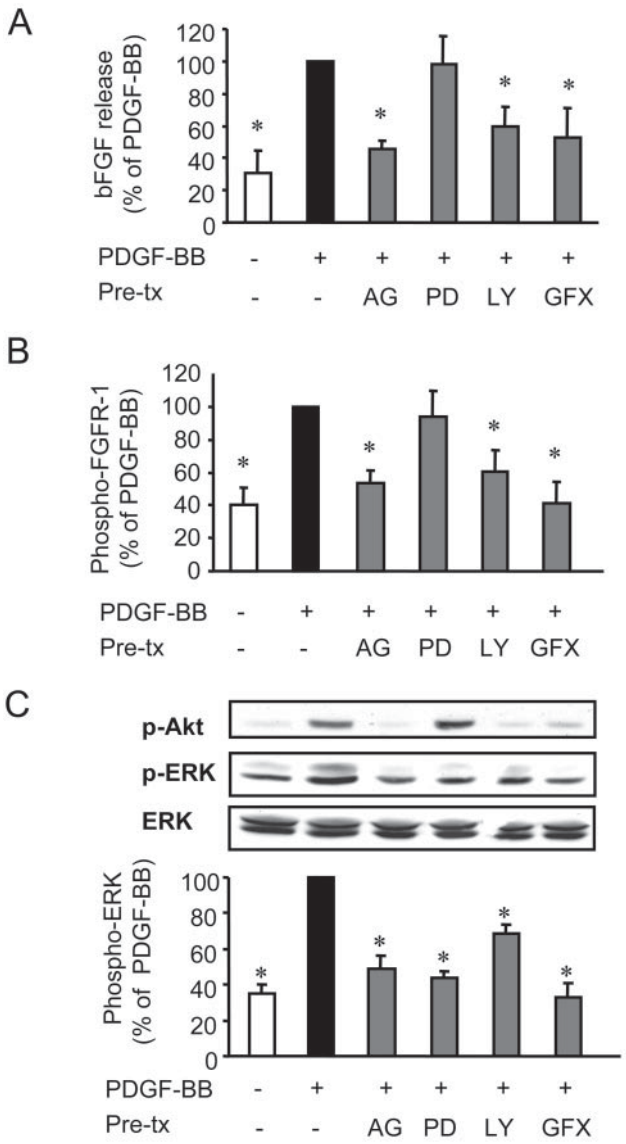

Figure 7. Signal transduction proteins involved in PDGF-BB–induced bFGF release (A), FGFR-1 phosphorylation (B), and prolonged ERK activity (C). Quiescent cells were stimulated with PDGF-BB (10 ng/mL) for 4 hours. Where indicated, AG1296 (AG, 20 μmol/L), PD98059 (PD, 40 μmol/L), LY-294002 (LY, 10 μmol/L), or GF109203X (GFX, 10 μmol/L) was added 30 minutes before PDGF-BB stimulation. Nonstimulated control was included (white bar). A, Basic FGF release. As described in Materials and Methods, the pericellular fraction of bFGF was determined by ELISA. B, FGFR-1 phosphorylation. Densitometric analysis of bands of FGFR-1 phosphorylation from 5 independent experiments was performed. C, Prolonged ERK activity. Representative blot (p-Akt, p-ERK, ERK) from 5 independent experiments with similar results is shown. Data (mean±SEM) are presented as percentage of control PDGF-BB stimulation. *$P \leq 0.05$ vs PDGF-BB stimulation (black bar).

(6.94±0.56 and 5.54±0.63 ng/10^6 cells without or with actinomycin D, respectively; mean±SEM; n=5; $P=0.175$), indicating that transcription is not required for PDGF-BB–mediated bFGF release.

Discussion

The novel finding in the present study is that sustained ERK activation and subsequent proliferation in response to PDGF-BB is partially mediated by endogenous basic FGF through activation of FGFR-1. It is known that entry into S phase requires ERK activity[1] and that sustained ERK activity is correlated with mitogenic activity.[6] We demonstrate that early activation of ERK by PDGF-BB is independent of bFGF, whereas sustained ERK activation depends on bFGF and FGFR-1. Treatment with bFGF antibody could be delayed for 4 hours without reducing its inhibitory effect, suggesting that bFGF is an autocrine "progression factor."[21] Our data further suggest that endogenous bFGF does not contribute to PI3-kinase activation by PDGF-BB because phosphorylation of the PI3-kinase substrate, Akt, is not affected by either bFGF blockade or FGFR-1 suppression.

To our knowledge, this is the first report showing that PDGF-BB induces activation of FGFR-1 and that FGFR-1 is required for a full mitogenic response to PDGF-BB. In rat aortic SMCs, FGFR-1 was shown to play an essential role in bFGF-induced effects.[20] Although our observation suggests a crucial role for FGFR-1 in bFGF-dependent, PDGF-BB–induced proliferation, we cannot rule out the contribution of additional members of the FGFR family.[22] FGFR-1 phosphorylation was observed as early as 30 minutes after PDGF-BB stimulation and the receptor remained phosphorylated after 4 hours. Because neutralizing bFGF antibody reduced PDGF-BB–induced mitogenesis and FGFR-1 phosphorylation, we conclude that PDGF-BB induces bFGF release.

We found that PDGF-BB significantly increases bFGF on the cell surface and the extracellular matrix, whereas the amount of bFGF in the media increases only slightly (Table). This result is in agreement with other studies showing that bFGF is bound to HSPGs on the cell surface and in the matrix.[14,23,24] Some of the HSPGs protect bFGF from degradation, whereas others on the cell surface are required for bFGF signaling.[25] Although syndecan-4 has been shown to increase bFGF-FGFR-1 signaling,[26,27] downregulation of syndecan-4 by siRNA does not interfere with PDGF-BB–induced proliferation in human SMCs (unpublished data, 2004). Because the inhibitory effect of bFGF antibody is lost when administration is delayed by 6 hours (Figure 4), we conclude that bFGF is required within the first 4 to 6 hours for prolonged ERK activity, which allows entry to S phase. However, because the largest increase of bFGF in the pericellular pool (from 2 to 4 hours; Figure 3) coincides with the late peak of bFGF-dependent ERK activation (from 2 to 8 hours; Figure 5), it is possible that, whereas late FGFR-1 activation depends on bFGF release, early FGFR-1 activation may depend on another mechanism, such as transactivation by the PDGF receptor.

Basic FGF lacks a traditional signal peptide for secretion and different mechanisms for its release have been proposed, including an integrin-dependent process, mechanical strain, membrane disruption, and the Na^+/K^+-ATPase.[23,28] Heparinase[24] and protease activity[29] can also release bFGF from the extracellular matrix. Our data demonstrate that PI3-kinase and protein kinase C, but not MAPK, are involved in bFGF release. This is, to our knowledge, the first observation of the requirement for PI3-kinase activity in bFGF release. FGFR-1 phosphorylation and prolonged ERK activity in response to

PDGF-BB also requires active PI3-kinase and protein kinase C. Interestingly, PI3-kinase mediates translocation of exogenous bFGF into the cell.[30] Although PDGF-BB is known to induce bFGF mRNA in rat SMCs,[31] PDGF-BB reduces bFGF mRNA in bovine SMCs.[32] We found in human SMCs that transcription is not necessary for PDGF-BB to increase proliferation through bFGF release. Further work will be required to elucidate whether PI3-kinase and PKC induce bFGF release through increased membrane permeability, Na^+/K^+-ATPase activity, FGF-binding protein availability,[33–36] or other mechanisms.

Whether bFGF contributes to PDGF-BB–induced migration of rat SMCs is uncertain.[31,37] Our experiments suggest that bFGF and FGFR-1 do not contribute to PDGF-BB–induced migration in human SMCs. This result is in accordance with our data showing that bFGF contributes to late, but not early, ERK activation in response to PDGF-BB.

Heparin suppresses rat SMC proliferation in vitro and in vivo,[38] in part by displacing bFGF from sites of injury.[39] In the present study, heparin inhibited PDGF-BB–induced ERK activation at 4 hours in normal and scrambled siRNA-treated cells but not in FGFR-1 downregulated cells. This result also suggests that heparin displaces bFGF from the cell surface, thus preventing binding to FGFR-1. However, heparin may also inhibit PDGF-BB–induced SMC proliferation by mechanisms unrelated to its interaction with bFGF. The variability of heparin responsiveness of human SMCs[40–42] may be of great clinical significance, because it has been reported that resistance to the inhibitory effect of heparin is related to increased risk of restenosis.[43] Whether heparin resistance is related to variations in bFGF signaling is an intriguing possibility.

In conclusion, the present study demonstrates that in human SMCs, maximal stimulation of proliferation by the tyrosine kinase receptor agonist PDGF-BB requires the sustained activation of ERK by another tyrosine kinase receptor, FGFR-1, which is activated by endogenous bFGF. This represents a novel mechanism that supports an important autocrine function of bFGF in human SMC proliferation.

Acknowledgments

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Arbeit #6

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Syndecan-4 Is Required for Thrombin-induced Migration and Proliferation in Human Vascular Smooth Muscle Cells*

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Thrombin is a mitogen and chemoattractant for vascular smooth muscle cells (SMCs) and may contribute to vascular lesion formation. We have previously shown that human SMCs, when stimulated with thrombin, release basic fibroblast growth factor (bFGF), causing phosphorylation of FGF receptor-1 (FGFR-1). Treatment with bFGF-neutralizing antibodies (anti-bFGF) or heparin inhibits thrombin-induced DNA synthesis. We concluded that thrombin may stimulate entry into the cell cycle via bFGF release and FGFR-1 activation. In the present study, we demonstrate a requirement for not only FGFR-1 but also syndecan-4, a transmembrane heparan-sulfate proteoglycan. Inhibition of syndecan-4 expression using small interfering RNA (siRNA) resulted in reduced DNA synthesis by human SMCs after stimulation with thrombin (10 nmol/liter). Anti-bFGF antibody, which inhibits DNA synthesis in control cells, had no inhibitory effect when syndecan-4 expression was reduced by siRNA. Thrombin- or bFGF-induced SMC migration, determined in Boyden chamber assays, was reduced in cells treated with syndecan-4 or FGFR-1 siRNA or by anti-bFGF. Thrombin induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in a biphasic pattern. Although thrombin-mediated ERK phosphorylation at 5 min was not affected by syndecan-4 or FGFR-1 siRNA, ERK phosphorylation at later time points was reduced. We conclude that thrombin-released bFGF binds to syndecan-4 and FGFR-1, which is required for thrombin-induced mitogenesis and migration.

Vascular smooth muscle cell (SMC)[1] proliferation and migration are key events in atherosclerosis and restenosis after vascular injury (1, 2). The G-protein-coupled receptor agonist thrombin may contribute to disease progression through stimulation of mitogenic signaling. In a previous study with cultured human SMCs, we demonstrated that thrombin releases basic fibroblast growth factor (bFGF), which is bound to the pericellular matrix and causes phosphorylation of the FGF receptor-1 (FGFR-1) (3). Preventing FGFR-1 phosphorylation by bFGF-neutralizing antibodies or by heparin inhibits thrombin-induced mitogenesis. Since heparin binds bFGF and inhibits bFGF-FGFR-1 interactions, we hypothesized that an endogenous heparan sulfate proteoglycan (HSPG) may be involved in mediating the effects of thrombin-released bFGF and thus be required for thrombin-induced mitogenesis.

Syndecan-4 is a transmembrane HSPG with a core protein of 35 kDa carrying mainly heparan sulfate side chains (4). Syndecans and the HSPG glypican have been shown to bind bFGF and increase bFGF-FGFR-1 interactions (5). Of the family of syndecans (syndecan-1 to -4), syndecan-4 is the only member with an intracellular domain that is capable of binding phosphatidylinositol 4,5-bisphosphate and protein kinase C α (PKCα), which allows it to support PKC-mediated signaling (6–8). Recently, several unique functions have been described for syndecan-4. It clusters in focal contacts and affects integrin function, thereby modulating cell adhesion, migration, and morphology (9–13). Since thrombin activates FGFR-1 by releasing bFGF into the pericellular matrix and syndecan-4 functions as a mediator for bFGF signaling and a cofactor for FGFR-1 (for a review, see Ref. 13), we tested the possibility that thrombin-induced mitogenesis and migration require syndecan-4 for binding thrombin-released bFGF.

EXPERIMENTAL PROCEDURES

Materials—Antibodies (Abs) against syndecan-4 and FGFR-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Ab against phosphorylated ERK1/2 was from Cell Signaling Technology (Beverly, MA). Ab to an HSPG neo-epitope, exposed after heparitinase III digestion (anti-ΔHS, clone 3G10), was from Seikagaku Corp. (Tokyo, Japan). Protein A-agarose was from Roche Diagnostics. Neutralizing Ab against human bFGF was a generous gift from Dr. Michael A. Reidy (University of Washington) (14). ERK1/2 antiserum was a generous gift from Dr. Karen Bornfeld (University of Washington). Human α-thrombin was from American Diagnostica (Greenwich, CT). Recombinant bFGF, heparin (porcine intestinal mucosa), and heparitinase I, II, and III were from Sigma. All cell culture solutions were from Invitrogen.

Cell Culture—Human aortic SMCs were prepared by the explant technique as described previously (15). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 200 units/ml penicillin, and 200 μg/ml streptomycin. Cells at passage 5–12 were used for experiments.

Gene Silencing with Small Interfering RNA (siRNA)—The following sequences were chosen to generate siRNA: for syndecan-4, 5′-AAGGCCGATACTTCTCCGGAG-3′ (sense), 5′-AAGGCTCTCCGGAG-CGATACT-3′ (scrambled); for FGFR-1, 5′-AAGTCGGACGCAACAGAGAAA-3′ (sense), 5′-AACAGAGAAAGTCGGACGCAA-3′ (scrambled). SiRNAs were generated *in vitro* using a siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions.

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[1] The abbreviations used are: SMC, vascular smooth muscle cell; FGF, fibroblast growth factor; bFGF, basic FGF; anti-bFGF, bFGF-neutralizing antibody; FGFR-1, FGF receptor-1; EGF, epidermal growth factor; HSPG, heparan sulfate proteoglycan; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKCα, protein kinase C α; Ab, antibody; siRNA, small interfering RNA; RT, reverse transcription; ANOVA, analysis of variance; HS, heparan sulfate.

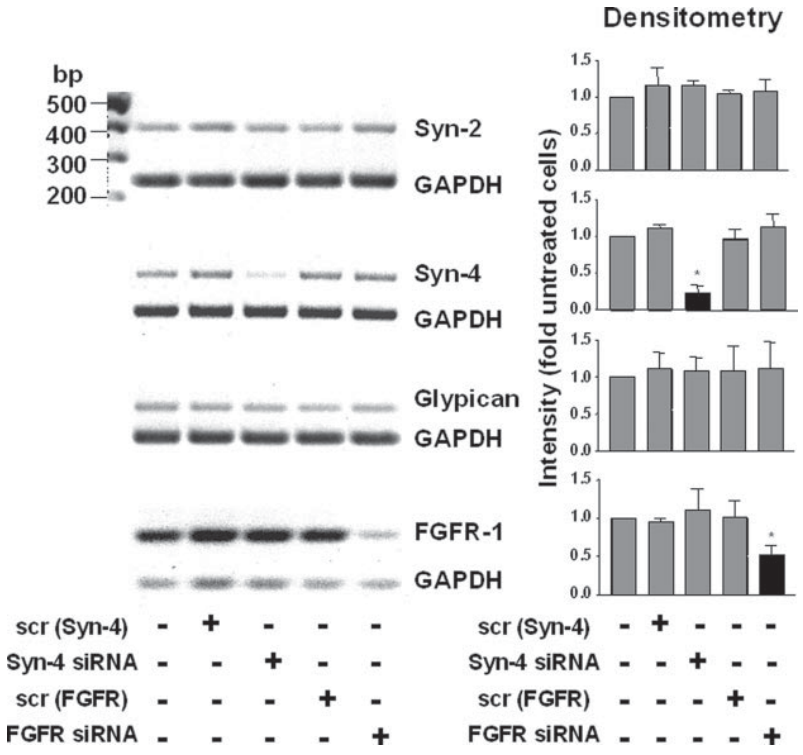

FIG. 1. **Inhibition of syndecan-4 (*Syn-4*) and FGFR-1 RNA by siRNA.** Cultured human vascular SMCs were either not treated or were transfected with siRNA-targeting syndecan-4 (*Syn-4 siRNA*) or FGFR-1 (*FGFR-1 siRNA*), or with matched scrambled RNA (*scr* (*Syn-4*) or *scr* (*FGFR-1*)). The RNA concentration (siRNA or scrambled RNA) used for transfections was 10 nmol/liter. After transfection, cells were starved for 48 h, and total RNA was isolated as described under "Experimental Procedures." RT-PCR was performed for syndecan-2 (*Syn-2*), syndecan-4, glypican, and FGFR-1 as described. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was co-amplified in each sample to serve as internal standard. The intensity of gene-specific bands is shown in relation to the respective glyceraldehyde-3-phosphate dehydrogenase signal and expressed as -fold over untreated cells. Values are mean ± S.E. of 3 independent experiments; *, $p < 0.05$ *versus* no treatment or scrambled RNA (ANOVA).

Cells were transfected with siRNAs (final concentration 10 nmol/liter) by calcium phosphate precipitation for 15 h, as described (16). Cells were washed three times with phosphate-buffered saline, once with medium containing 15% fetal bovine serum, and allowed to recover for at least 9 h. Cells were detached with trypsin, counted, and reseeded for experiments in 15% serum.

Immunoprecipitation and Western Blotting—Immunoprecipitation of FGFR-1 was carried out as described previously (3). For detection of syndecan-4 or HSPG neo-epitope (ΔHS), cells were lysed with 8 M urea buffer (25 mmol/liter Tris-HCl, 8 M urea, pH 7.5, 2 mmol/liter phenylmethylsulfonyl fluoride). Cell lysates were frozen at −80 °C to disrupt syndecan-4 dimers (13). Cell extracts were dialyzed against 0.1 mol/liter sodium acetate, pH 7.0, 0.1 mmol/liter calcium acetate. Samples were concentrated using centrifugal filter tubes (Amicon®, cut-off 10,000, Millipore, MA) and resuspended in 100 μl of digestion buffer (10 mmol/liter calcium acetate, 18 mmol/liter sodium acetate, 0.1 M Tris-HCl, pH 7.4, 2 mmol/liter phenylmethylsulfonyl fluoride). Samples were incubated with heparitinase I–III (10 units/ml heparitinase I and II, 2.5 units/ml heparitinase III) for 4 h at 37 °C to digest HS side chains. Protein concentrations were determined to ensure equal protein loading. To determine ERK phosphorylation, cells were seeded into 6-well plates (80,000–100,000 cells/well) and incubated in serum-free medium for 48 h with a change of medium 24 h before stimulation.

For Western blot analysis, samples were boiled in Laemmli sample buffer and subjected to SDS-PAGE followed by transfer to nitrocellulose. Primary antibodies used were monoclonal Ab against syndecan-4 (clone 5G9, Santa Cruz Biotechnology), anti-ΔHS (clone 3G10, Seikagaku Corp.), anti-ERK1/2 (Cell Signaling), anti-actin (Sigma), and anti-FGFR1 (Santa Cruz Biotechnology). Protein was visualized by enhanced chemiluminescence (kit from Amersham Biosciences) according to the manufacturer's protocol. Results were quantified by densitometry of films using ImageQuant software (Amersham Biosciences).

Semiquantitative RT-PCR—Untreated SMCs or those transfected with 10 nmol/liter siRNA were seeded into 100-mm dishes ($5–6 \times 10^5$ cells/dish) and incubated in serum-free medium for 48 h. RNA was prepared using RNeasy® spin columns (Qiagen, Valencia, CA). Semiquantitative RT-PCR was performed with Ready-To-Go™ RT-RCR Beads (Amersham Biosciences) using 1 μg of total RNA and 15 pmol of primer according to the manufacturer's instructions. Primer pairs, product size, and annealing temperature were as follows: FGFR-1, sense, 5′-CGGTGTGCCTGTGGAGGAACTT-3′, antisense, 5′-GTTACAGCTGACGGTGGAGTCT-3′ (408-bp fragment, 65 °C) (17); syndecan-2, sense, 5′-GGGAGCTGATGAGGATGTAG-3′, antisense, 5′-CACTGGATGGTT-TGCGTTCT-3′ (394-bp fragment, 60 °C); syndecan-4, sense, 5′-CTCCTAGAAGGCCGATACTTCT-3′, antisense, 5′-GGACCTCCGTTCTCT-CAAAGAT-3′ (345-bp fragment, 60 °C); glypican, sense, 5′-ATCACCGACAAGTTCTGGGGTA-3′, antisense, 5′-CATCTTCTCACTGCACA-GTGTC-3′ (317-bp fragment, 60 °C) (18). For semiquantitative analysis, glyceraldehyde-3-phosphate dehydrogenase was co-amplified: sense, 5′-TGATGACATCAAGAAGGTGGTGAA-3′, antisense, 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ (238-bp fragment, 60–65 °C) (19). Conditions for PCR after reverse transcription were: 95 °C for 1 min, specific annealing temperature for 1 min, 72 °C for 2.5 min, and a final elongation step at 72 °C for 15 min.

DNA Synthesis—SMCs (7,500–8,750 cells/cm^2) were incubated in serum-free medium for 48 h with a change of medium 24 h before stimulation. Cells were stimulated by the addition of thrombin (10 nmol/liter) or bFGF (1 ng/ml). [^{3}H]Thymidine (1 μCi/ml) was added 16–18 h after stimulation. After 26–28 h, cells were washed three times with ice-cold phosphate-buffered saline followed by incubation in 10% trichloroacetic acid overnight at 4 °C. Cells were washed in trichloroacetic acid, and DNA was solubilized in 0.1 N NaOH. Radioactivity was measured by liquid scintillation counting.

Boyden Chamber Migration Assay and Cell Spreading—Modified Boyden chamber assays were performed for 5 h at 37 °C with 48-well microchemotaxis chambers (Neuro Probe) and polycarbonate filters (10-μm pores; Nucleopore Corp.) coated with monomeric collagen (100 μg/ml Vitrogen 100 in 0.1 mol/liter acetic acid, Collagen Corp., Palo Alto, CA), as described (15). Cells (20,000/well) were added to the upper chamber, and chemoattractants or serum-free medium were added to the lower chamber. Migration was measured as the number of cells/high power field (×100) that had migrated across the membrane. Assays were performed in quadruplicate. For cell spreading experiments, cells were seeded on glass slides coated with monomeric collagen (100 μg/ml Vitrogen 100 in 0.1 mol/liter acetic acid) and allowed to spread for up to 6 h in serum-free medium. Cells were fixed and stained with Diff-Quick staining solution (Baxter, Detroit, MI) according to the manufacturer's instructions.

Statistics—All experiments were performed at least three times in duplicate, triplicate, or quadruplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test or by a paired two-tailed *t* test as indicated. Values of $p < 0.05$ were considered significant.

RESULTS

Inhibition of Syndecan-4 and FGFR-1 Expression by siRNA in Human Vascular SMCs—Specific siRNA significantly reduced RNA levels for syndecan-4 and FGFR-1 in human SMCs, respectively, but had no effect on RNA of syndecan-2 or glypi-

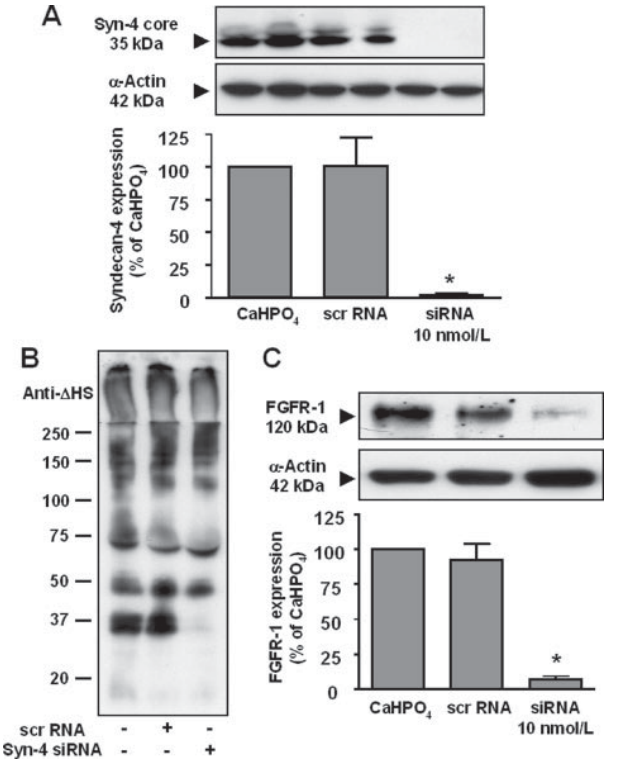

FIG. 2. **Inhibition of syndecan-4 (*Syn-4*) and FGFR-1 protein expression by siRNA.** Syndecan-4 core protein and FGFR-1 were determined by Western blotting as described under "Experimental Procedures"; α-actin in total cell lysates is shown for loading control. *A*, the effect of 10 nmol/liter siRNA to syndecan-4 on syndecan-4 protein. The blot contains duplicates of each sample. *B*, the effect of 10 nmol/liter siRNA to syndecan-4 on cell layer-bound HSPGs. HSPGs were isolated and digested with heparitinase as described. HS stubs antibody (anti-ΔHS, clone 3G10) was used to detect HSPG core proteins. A similar pattern with bands of ~35–37, 48, and 70 kDa and some high molecular bands has been described by others (20). The 35–37-kDa band presumably corresponds to syndecan-4 core protein. *scr RNA*, scrambled RNA. *C*, the effect of 10 nmol/liter siRNA to FGFR-1 on FGFR-1 protein. Values are mean ± S.E. of 4–5 independent experiments; *, $p < 0.05$ *versus* $CaHPO_4$ and scrambled RNA (ANOVA).

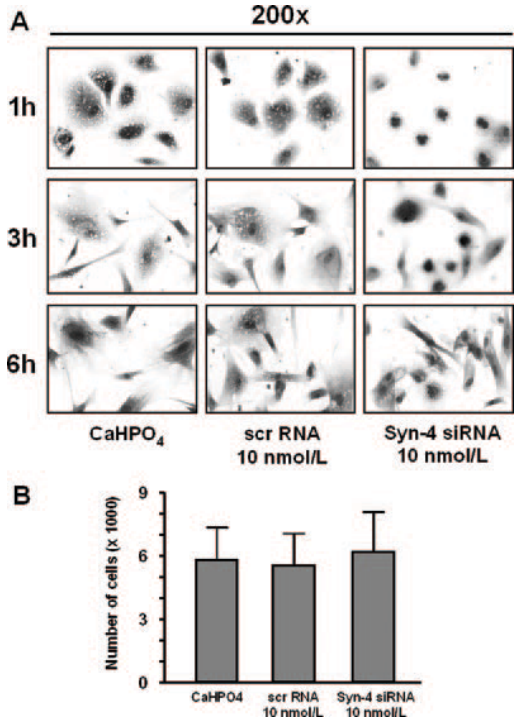

FIG. 3. **Cell spreading, but not adhesion, is impaired in SMCs with reduced syndecan-4 expression.** *A*, SMCs were seeded in serum-free medium on glass slides coated with monomeric collagen. Cells, treated with $CaHPO_4$ alone, with syndecan-4 siRNA (*Syn-4 siRNA*), or with matched scrambled RNA (*scr RNA*), were allowed to spread for 1, 3, or 6 h. Pictures are shown at ×200 magnification after fixation and staining. *B*, SMCs (10,000/well) were seeded in 12-well plates overnight in 15% serum, and cells were washed with phosphate-buffered saline, detached with trypsin, and counted. Values are mean ± S.E. of 4 independent experiments.

can, two proteoglycans that can both bind bFGF (5). RNA oligonucleotides with a scrambled sequence derived from syndecan-4 or FGFR-1 siRNA had no effect on RNA levels (Fig. 1).

Syndecan-4 protein levels were determined in extracts from human aortic SMCs by Western blotting. Although the specific siRNA inhibited syndecan-4 protein expression by over 90%, a matched scrambled RNA oligonucleotide had no effect on syndecan-4 expression (Fig. 2*A*). To determine whether other HSPGs in the cell layer were affected by syndecan-4 siRNA, an antibody (anti-ΔHS, clone 3G10) that detects an HSPG neoepitope after heparitinase digestion was used on Western blots of SMC lysates prepared as described under "Experimental Procedures" (Fig. 2*B*). Multiple bands, similar to those described by others using this antibody (20), were detected. A double band of 35–37 kDa, presumably syndecan-4 core protein, was strongly reduced in cells treated with syndecan-4 siRNA. These data indicate that whereas other HSPGs are present, only syndecan-4 is altered by siRNA to syndecan-4. An effective siRNA was also generated to inhibit FGFR-1, as determined in immunoprecipitates from SMC lysates (Fig. 2*C*).

Cell Spreading Is Impaired in SMCs with Reduced Syndecan-4 Expression—Because syndecan-4 is involved in the organization of the cytoskeleton (9, 21), we investigated whether syndecan-4 siRNA affects cell spreading. When compared with controls, syndecan-4 siRNA-treated cells spread less at 1 and 3 h in serum-free medium. However, by 6 h, all cells were equally spread on collagen (Fig. 3). Cell spreading in 15% serum appeared to be normal, and also, the number of cells that attached in 15% serum overnight was not decreased in syndecan-4 siRNA cells (Fig. 3*B*) when compared with control cells. These data suggest that the lack of syndecan-4 delays cell spreading on collagen. Delayed spreading was not observed in cells with decreased FGFR-1 expression, indicating distinct functions of syndecan-4 and FGFR-1.

Syndecan-4 and FGFR-1 Are Required for Thrombin- and bFGF-induced Migration—In Boyden chamber assays, thrombin- or bFGF-induced migration of SMCs transfected with syndecan-4 siRNA was impaired when compared with the migration of scrambled RNA-treated control cells (Fig. 4*A*). In contrast, serum-induced migration was not affected by syndecan-4 siRNA. SMCs transfected with FGFR-1 siRNA also migrated less toward thrombin than control cells, and unlike control cells, bFGF-neutralizing antibodies had no inhibitory effect on thrombin-induced migration (Fig. 4*B*). The migration pattern of untreated SMCs was not different from control cells treated with scrambled RNA matched for either syndecan-4 or FGFR-1 siRNA (data not shown).

Syndecan-4 and FGFR-1 Are Required for Thrombin-induced bFGF-dependent DNA Synthesis—SMCs transfected with syndecan-4 or FGFR-1 siRNA or with matched scrambled RNA were stimulated with thrombin or bFGF. In cells treated with syndecan-4 siRNA, DNA synthesis in response to thrombin or bFGF was reduced (Fig. 5*A*). A bFGF-neutralizing antibody inhibited thrombin-induced DNA synthesis in control cells but had no inhibitory effect when syndecan-4 expression was reduced by siRNA. Also of interest, inhibition of thrombin-induced DNA synthesis by heparin was only significant in control

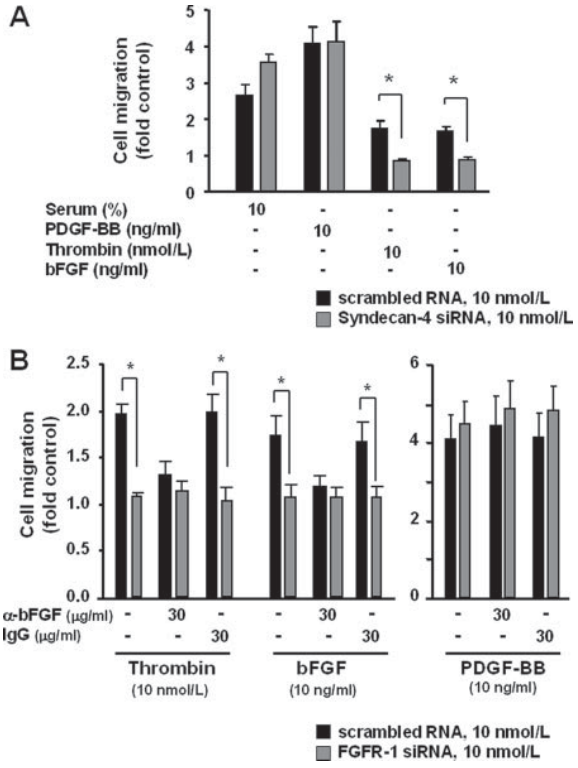

FIG. 4. **Syndecan-4 and FGFR-1 are required for thrombin- and bFGF-induced migration.** SMCs were transfected with siRNA (*gray bars*) or with matched scrambled RNA (*black bars*). 48 h after transfection, Boyden chamber assays were performed in 48-well chambers. After 6 h of stimulation, filters were removed from the chambers, and cells on the lower side of the filters were stained. The cell number is shown as -fold over non-stimulated controls. *A*, in cells treated with syndecan-4 siRNA or matched scrambled RNA, migration was induced with serum (10%), thrombin (10 nmol/liter), or bFGF (10 ng/ml). *B*, in cells treated with FGFR-1 siRNA or matched control RNA, migration was induced with thrombin (nmol/liter) and bFGF (10 ng/ml) in the absence or presence of bFGF-neutralizing antibodies (α-*bFGF*, 30 μg/ml) or nonspecific IgG (30 μg/ml). Values are mean ± S.E. of 5–6 independent experiments; *, $p < 0.05$ (paired two-tailed *t* test).

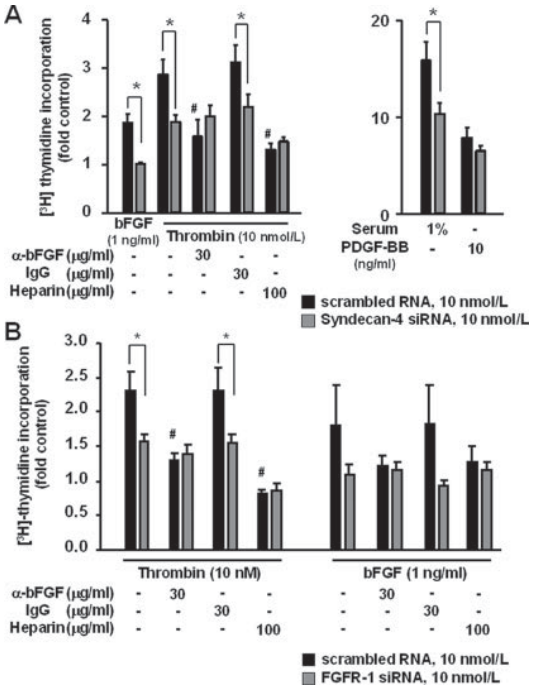

FIG. 5. **Syndecan-4 and FGFR-1 are required for thrombin-induced bFGF-dependent DNA synthesis.** SMCs were transfected with siRNA (*gray bars*) or with matched scrambled RNA (*black bars*) as described under "Experimental Procedures." *A*, SMCs transfected with syndecan-4 siRNA or matched scrambled RNA were stimulated with bFGF (1 ng/ml) or thrombin (10 nmol/liter) in the absence or presence of anti-bFGF (α-*bFGF*, 30 μg/ml), nonspecific IgG (30 μg/ml), or heparin (100 μg/ml). *B*, SMCs transfected with FGFR-1 siRNA or matched scrambled RNA were stimulated with thrombin (10 nmol/liter) or bFGF (1 ng/ml) in the absence or presence of anti-bFGF, IgG, or heparin. Values are mean ± S.E. of 7 (in *A*) or 4 (in *B*) independent experiments; *, $p < 0.05$ for siRNA *versus* scrambled RNA, #, $p < 0.05$ for cells pretreated with anti-bFGF or heparin *versus* thrombin alone (ANOVA).

cells. A nonspecific IgG had no effect in either experimental group (Fig. 5*A*).

The effects of reduced FGFR-1 expression were similar to those observed with reduced syndecan-4 expression. Thrombin-induced DNA synthesis was inhibited in cells with reduced FGFR-1 expression, and bFGF-neutralizing antibodies exerted no further inhibitory effect (Fig. 5*B*). Heparin significantly inhibited DNA synthesis only in control cells. In contrast to control cells, stimulation with recombinant bFGF of cells treated with FGFR-1 siRNA did not result in increased DNA synthesis.

Late Phase of Thrombin-induced ERK1/2 Phosphorylation Requires Syndecan-4 and FGFR-1—To determine whether ERK 1/2 is involved in syndecan-4- and FGFR-1-controlled intracellular pathways that are activated by thrombin, non-treated SMCs and SMCs transfected with scrambled RNA, syndecan-4 siRNA, or FGFR-1 siRNA were stimulated with thrombin from 5 min to 4 h. The extent of ERK phosphorylation at 5 min in control cells and siRNA cells was the same, whereas at later time points, it was reduced in SMCs transfected with either syndecan-4 siRNA (Fig. 6*A*) or FGFR-1 siRNA (Fig. 6*B*).

DISCUSSION

In previous studies, we and others have shown that thrombin-induced mitogenesis and migration require the activation of a secondary ligand-receptor system (15, 22). In rat SMCs, thrombin transactivates the EGF receptor by releasing heparin-binding EGF-like growth factor (HB-EGF) from the cell surface (15). In contrast, we recently demonstrated that in human SMCs, thrombin does not transactivate the EGF receptor. Instead, thrombin causes rapid release of bFGF into the pericellular matrix with subsequent FGFR-1 phosphorylation, which can be blocked by bFGF-neutralizing antibodies and by heparin (3). In the present study, we have demonstrated that the cell surface heparan sulfate proteoglycan syndecan-4 is required for thrombin-induced mitogenesis and migration by using syndecan-4 siRNA to specifically decrease syndecan-4 core protein (Fig. 2*B*). We found that human SMCs with reduced syndecan-4 or FGFR-1 migrate and proliferate less in response to thrombin or bFGF (Figs. 4 and 5). The magnitude of this effect is similar to the level of inhibition of thrombin-mediated mitogenesis obtained by treating normal cells with bFGF-neutralizing antibodies (3). In contrast, the bFGF-neutralizing antibody did not alter thrombin- or bFGF-mediated migration and proliferation of FGFR-1 knockdown SMCs. These data indicate that the thrombin-induced bFGF-dependent pathway is mediated by both syndecan-4 and FGFR-1.

Syndecan-4, as well as syndecan-1 and -2 and glypicans, can bind bFGF and increase bFGF-FGFR-1 interactions (5). In addition, syndecan-4 is known to play a unique role in bFGF-dependent signal transduction (9, 13, 23). The cytoplasmic tail of syndecan-4 forms a complex with phosphatidylinositol 4,5-bisphosphate and PKCα, which promotes PKCα activation (6–8). It has been proposed that a serine/threonine protein phosphatase becomes activated after bFGF binds to its tyrosine kinase receptor. This phosphatase dephosphorylates the cytoplasmic tail of syndecan-4, increasing its affinity for phosphati-

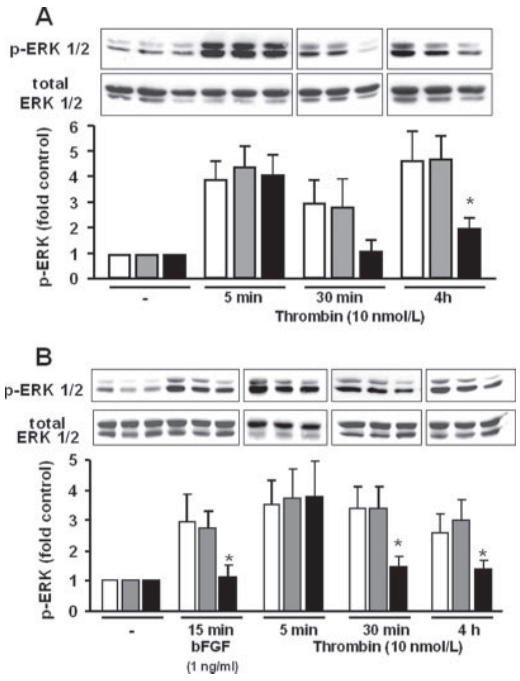

FIG. 6. **Late phase of thrombin-induced ERK1/2 phosphorylation requires syndecan-4 and FGFR-1.** *A* and *B*, non-treated SMCs (*white bars*), SMCs transfected with syndecan-4 siRNA (*A*) (*black bars*) or FGFR-1 siRNA (*B*) (*black bars*) or with the respective matched scrambled RNA (*gray bars*) were seeded in 6-well plates overnight and then incubated in serum-free medium for 48 h with a medium change 24 h before stimulation. Cells were stimulated with thrombin (10 nmol/liter) or bFGF (1 ng/ml) for the indicated times and lysed with Laemmli sample buffer. ERK 1/2 phosphorylation was determined with phospho-specific ERK antibody (*p-ERK 1/2*). Blots were stripped and reprobed for ERK1/2. Densitometry values are mean ± S.E. of 4 independent experiments and are shown as -fold over non-stimulated controls; *, $p <$ 0.05 for siRNA *versus* non-treated cells and scrambled RNA (ANOVA).

dylinositol 4,5-bisphosphate and promoting dimerization (and possibly higher order multimers) of the syndecan-4 cytoplasmic tail. This in turn increases the binding and activation of PKCα (24). It is known that thrombin-induced signaling involves activation of PKC isoforms and translocation of PKCα into focal domains (25). Therefore, since syndecan-4 activates PKCα (6–8), it is possible that thrombin may exert its effects on migration and proliferation, in part, via promoting bFGF and syndecan-4 dependent PKCα activation.

Thrombin also induces signal transduction through the MAPK pathway, and prolonged ERK activation is required for cell cycle progression induced by various mitogens, including thrombin (26). In rat SMCs, the EGF receptor co-stimulatory pathway was required to sustain ERK activity beyond 30 min (15). In human SMCs, we found that this sustained activation of ERK by thrombin required syndecan-4 and FGFR-1 (Fig. 6), in part explaining the role of syndecan-4 and FGFR-1 in thrombin-mediated SMC proliferation (Fig. 5). It would be of interest to further investigate the mechanisms involved in syndecan-4 dependent bFGF-mediated ERK activation.

Our observation of impaired spreading of syndecan-4 siRNA cells on collagen-coated slides (Fig. 3) is in agreement with previous reports that syndecan-4 is involved in cell spreading through the assembly of focal contacts (9, 13). This may explain the reduced migration of these SMCs (Fig. 4). Although we did not directly investigate focal adhesion formation, the observation that syndecan-deficient cells were fully spread by 6 h suggests that human SMCs have sufficient compensatory mechanisms to form focal adhesions. This is consistent with observations using fibroblasts from syndecan-4-deficient animals. Although these cells are able to form normal focal adhesions (13, 27), focal adhesion formation is impaired when the cells are cultured on the cell binding fragment of fibronectin in the presence of culture medium with the heparin-binding fragment of fibronectin (27). Of interest, these syndecan-4-deficient mice develop normally, but when challenged, they exhibit impaired wound healing and decreased angiogenesis in the granulation tissue (28, 29). Thus, the specific role of syndecan-4 for focal adhesion formation, especially in human cells, is uncertain and requires further study.

In summary, we demonstrate a requirement for syndecan-4 and FGFR-1 for thrombin-induced bFGF-dependent migration, mitogenesis, and sustained ERK activation in human vascular SMCs.

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Arbeit #7

Rauch BH, Scholz GA, Baumgärtel-Allekotte D, Censarek P, Fischer JW, Weber AA, Schrör K.

Cholesterol Enhances Thrombin-induced Release of Fibroblast Growth Factor-2 in Human Vascular Smooth Muscle Cells.

Arterioscler Thromb Vasc Biol 2007; 27:e20-e25.

Rapid Communication

Cholesterol Enhances Thrombin-Induced Release of Fibroblast Growth Factor-2 in Human Vascular Smooth Muscle Cells

Bernhard H. Rauch, Godehard A. Scholz, Dana Baumgärtel-Allekotte, Petra Censarek, Jens W. Fischer, Artur-Aron Weber, Karsten Schrör

Objective—The mitogenic response to the G protein–coupled receptor agonist thrombin in human vascular smooth muscle cells (SMCs) depends on release of fibroblast growth factor-2 (FGF-2). Yet, intracellular mechanisms triggering FGF-2 release are unknown. The present study investigates possible effects of cholesterol enrichment and depletion, which have been shown to influence FGF-2–dependent signaling and SMC mitogenesis, on thrombin-induced FGF-2 release.

Methods and Results—Cultured human aortic and saphenous vein SMCs were enriched with cholesterol by using a cyclodextrin-cholesterol complex. Cholesterol accumulation was determined by a fluorometric assay. ELISA, Western blotting, and RT-PCR were used for quantification of FGF-2 levels. DNA synthesis was determined by [^{3}H]-thymidine incorporation, proliferation by cell counting. Stimulation of SMCs with thrombin (30 nmol/L) resulted in release of FGF-2 into the pericellular space within 10 minutes. Preincubation with cyclodextrin-cholesterol caused accumulation of cellular cholesterol, increased thrombin-induced FGF-2 release, and stimulated FGF-2 de novo synthesis. Thrombin-induced DNA synthesis and proliferation were enhanced in cholesterol-rich SMCs. This effect was inhibited by FGF-2-neutralizing antibodies.

Conclusions—Enhanced cellular cholesterol stimulates thrombin-induced release of FGF-2 and increases the mitogenic response toward thrombin in human SMCs. This mechanism might also be relevant for thrombin-induced mitogenesis in hypercholesterolemia in vivo. **(*Arterioscler Thromb Vasc Biol*. 2007;27:e20-e25.)**

Key Words: thrombin ■ FGF-2 ■ cholesterol ■ smooth muscle cells ■ mitogenesis

Proliferation of vascular smooth muscle cells (SMCs) is a key event in atherosclerosis and restenosis after vascular injury.[1,2] In addition to its function as coagulation factor, thrombin acts as a mitogen for SMCs by activating the G protein–coupled protease-activated receptors (PARs).[3] In previous studies, we and others have shown that the mitogenic signaling of G protein–coupled receptors (GPCRs) involves transactivation of receptor-tyrosine kinases (RTK).[4–6] In human SMCs, we found that thrombin-induced mitogenesis depends on a rapid release of fibroblast growth factor-2 (FGF-2) into the pericellular matrix and consecutive activation of the FGF receptor-1 (FGFR-1).[6] The role of FGF-2 as a mitogen and chemoattractant for SMCs in vitro and in vivo is well characterized.[7–11] Interestingly, it is still unknown how FGF-2 is released from intact cells. Because FGF-2 lacks a typical amino acid sequence for externalization,[12] earlier studies speculated that FGF-2 may be released on cell damage or by an exocytotic mechanism independent of the endoplasmic reticulum–Golgi pathway.[13]

Elevated non–high density lipoprotein (non-HDL) cholesterol levels are not only an established risk factor for the development and progression of atherosclerosis, but have also been correlated with complications of the disease, for example restenosis after percutaneous transluminal coronary angioplasty (PTCA) and bypass surgery.[14–16] In experimental studies performed in rat and rabbit SMCs, enrichment of SMCs with cholesterol has been shown to increase the mitogenic response to growth factors (PDGF-BB)[17] or to induce synthesis of the RTK ligand FGF-2.[18] However, whether elevated cholesterol levels also affect the mitogenic response toward the GPCR ligand thrombin in vascular SMCs has not yet been studied. In a recent study, a complex of methyl-β-cyclodextrin (MbCD) and cholesterol has been used to enrich cellular cholesterol levels.[19] After treatment of SMCs with MbCD-cholesterol, a foam cell-like transformation of SMCs has been described, indicating that this method may be useful to increase cholesterol-uptake in SMCs.[19] In addition, treat-

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ment of cells with MbCD has been described to deplete cell membranes from cholesterol. This results in increased cell membrane stiffness[20] and disturbed FGF-2 signaling.[21] These effects of MbCD were antagonized and intracellular cholesterol was enriched by exposing the cells to MbCD saturated with cholesterol.[20,21]

The present study investigates the impact of cholesterol enrichment and depletion on thrombin-induced release of FGF-2 and cell function in human vascular SMCs. In addition to cultured aortic SMCs, saphenous vein SMCs were also used because these cells are widely used as bypass graft material. Our data demonstrate for the first time that accumulated cellular cholesterol enhances thrombin-dependent mitogenesis in human SMCs and does so via increased release of FGF-2.

Materials and Methods

Materials

Methyl-β-cyclodextrin (MbCD)-cholesterol-complex, cholesterol-free MbCD, heparin, and β-actin antibodies (abs) were from Sigma-Aldrich (München, Germany). Abs against extracellular-regulated kinase (ERK1/2) were from New England Biolabs (Frankfurt, Germany). Neutralizing abs against human basic fibroblast growth factor (FGF-2) and nonspecific control IgG were a generous gift from Dr Michael A. Reidy (University of Washington, Seattle). Purified α–thrombin was kindly provided by Dr Jörg Stürzebecher (Friedrich-Schiller-Universität, Jena, Germany). Specific PAR-activating peptides (PAR-AP) were from Biosyntan (Berlin, Germany). GF109203X, PD98059, genistein, BAPTA/AM, colchicin, and adenylyl-imidodiphosphate were from Calbiochem (Darmstadt, Germany).

Cell Culture

Human SMCs were prepared from aorta or saphenus veins by the explant technique and cultured as described previously.[6,22] Cells were synchronized by serum deprivation for 48 hours before the experiments.

Cholesterol-Enrichment of Human SMC

Cellular cholesterol was stained by oil-red O, as described by others.[19] Nuclei were stained with hemalaun. Images were taken with an Olympus BX 50 microscope connected to a colorview II camera (Soft Imaging System) as described.[23] Intracellular concentrations of cholesterol were determined after incubation of SMCs with MbCD-cholesterol (10 μg/mL cholesterol) or MbCD for 24 hours with the Amplex red cholesterol assay kit (Invitrogen) according to the manufacturer's instructions. Fluorescence was determined at 590 nm using a Fluoroskan Ascent microplate reader (Thermo Labsystems).

Enzyme-Linked Immunosorbent Assay

Basic FGF was determined in the media, the pericellular matrix, and intracellular by using an ELISA (R&D Systems) according to the manufacturer's instructions. After stimulation, medium was removed and cells were incubated for 20 minutes at room temperature on a rocking shaker with PBS containing 10 μg/mL heparin. Cells were detached with trypsin/EDTA, counted, then pelleted and lysed with lysis buffer (25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1% 2-mercaptoethanol, 1% Triton-X100, 2 mmol/L PMSF) as described.[6] Medium, heparin wash, and cell lysates were stored at −80°C.

Semiquantitative RT-PCR and Real-Time PCR

Semiquantitative RT-PCR was performed using OneStep RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Primer pairs for FGF-2 were: sense, 5′-CTTCTTCCTGCGCATCCAC-3′, antisense, 5′-TCAGCTCTTAGCAGACATTG-3′ (354-bp fragment, 56°C annealing temperature). Primer pairs for internal GAPDH control were as described previously.[24] Quantitative PCR was performed on a Real-Time PCR System 7300 (Applied Biosystems). TaqMan gene expression assays Hs00266645 (FGF-2) and Hs99999905 (internal GAPDG control) were used according to the manufacturer's instructions. Data were analyzed with the Sequence Detection Software v1.2.3.

Immunoblotting

Immunoblotting was performed as described previously.[6,24]

DNA Synthesis and Cell Count

DNA synthesis was determined by [^{3}H]-thymidine incorporation, proliferation by cell count as described.[6,25]

Statistics

Data are mean±SEM of n independent experiments as indicated. Statistical analysis was performed using one-way ANOVA followed by Bonferroni multiple comparison test. Probability values of <0.05 were considered significant.

Results and Discussion

Human saphenous vein SMCs were incubated with thrombin (30 nmol/L) for 5 to 60 minutes (Figure 1A). A maximum release of FGF-2 to the pericellular matrix was seen after 10 to 15 minutes of stimulation, and control levels were observed after 1 to 24 hours (not shown). In contrast, FGF-2 concentrations in the culture media were unchanged over the time of incubation with thrombin (not shown). This is in agreement with previous data in human aortic SMCs.[6] In addition to our previous study, specific PAR-activating peptides (PAR-AP) were used to evaluate which PARs may be involved in thrombin-induced FGF-2 release. Although RNA for all PARs is expressed in these cells (not shown), only PAR1- and PAR3-AP increased the release of FGF-2 into the pericellular matrix (Figure 1B). PAR4-AP had a similar effect, whereas PAR2-AP was ineffective (Figure 1B). This is consistent with the observation that thrombin activates only PAR1, -3, and -4 but not PAR2.[26] Of note is that PAR3-AP may not be specific for PAR3. A recent report suggests that it can also activate PAR1.[27]

Human vascular SMCs were enriched with cholesterol by incubating them with MbCD-cholesterol (Figure 2). This method has been reported to induce cholesterol accumulation and to cause transformation of mouse SMCs into a foam cell–like phenotype after incubation for several days.[19] In human SMCs, the morphology of the cells after incubation with MbCD-cholesterol for 24 hours appeared rather unchanged, except for a vesicular staining with oil-red-O indicating the excessive cholesterol (Figure 2). Intracellular cholesterol concentrations were increased significantly from 2.21±0.17 μg/mL in control cells to 3.88±0.27 μg/mL (n=6) after incubation with MbCD-cholesterol. In contrast, treatment with MbCD has been reported to extract cholesterol from cells and to increase cell membrane stiffness.[20] In agreement with this report, MbCD significantly reduced cellular cholesterol levels (to 1.66±0.10 μg/mL). The intracellular cholesterol contained about 20% cholesterol-ester, whereas 80% were free cholesterol in all groups (data not shown).

To assess effects of MbCD-cholesterol and MbCD on thrombin-dependent FGF-2 release, both human saphenous

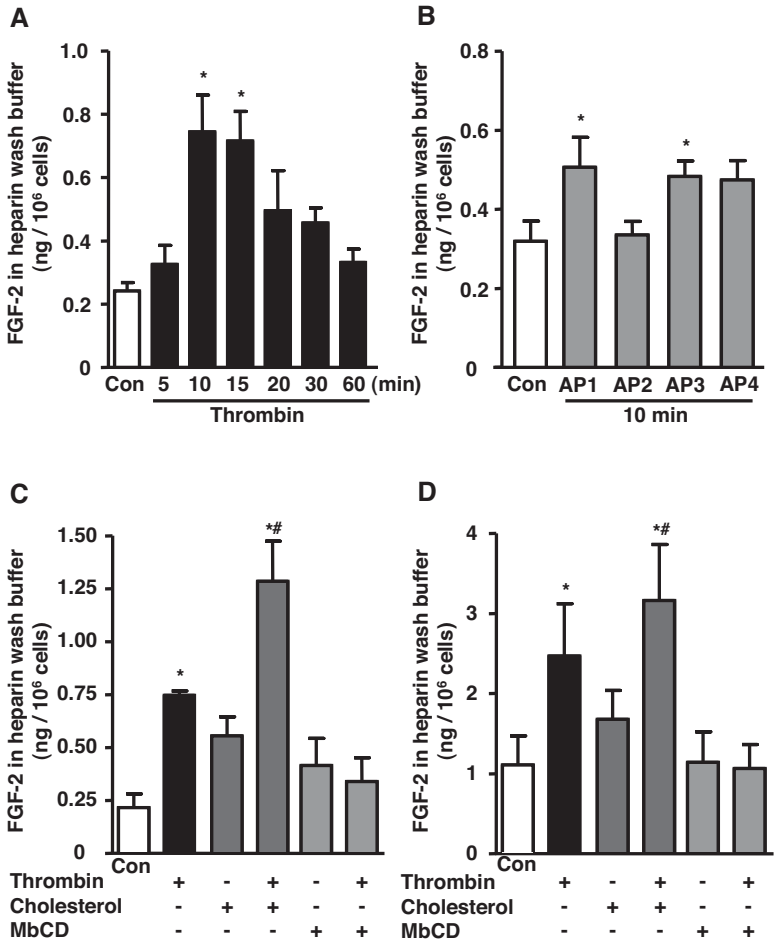

Figure 1. Thrombin-induced release of FGF-2 into the extracellular matrix is modulated by cholesterol in human SMCs. FGF-2 was removed from the cell layer by washing with heparin-containing PBS and determined by ELISA. Human saphenous vein SMCs were stimulated with thrombin (30 nmol/L) for the indicated times (A) or with specific PAR-activating peptides (AP1–4, 100 μmol/L each) for 10 minutes (B); mean±SEM of 5 (A) and 4 (B) independent experiments, $^{*}P<0.05$ vs controls. Both human saphenous vein (C) and aortic SMCs (D) were treated with either MbCD-cholesterol (10 μg/mL cholesterol) or the equivalent amount of MbCD for 24 hours. After stimulation with thrombin (30 nmol/L) for 10 minutes, release of FGF-2 into the pericellular matrix was determined by ELISA (mean±SEM of 4 independent experiments, $^{*}P<0.05$ vs control, $^{\#}P<0.05$ vs cholesterol).

vein and aortic SMCs were stimulated with thrombin for 10 minutes after incubation with MbCD-cholesterol or MbCD for 24 hours. Pretreatment with MbCD-cholesterol increased thrombin-released FGF-2 in both cell types, whereas MbCD inhibited thrombin-induced release of FGF-2 (Figure 1C and 1D). Cell numbers were not affected by pretreatment with either MbCD or MbCD-cholesterol (not shown). These data suggest that accumulated intracellular cholesterol increases FGF-2 release, whereas MbCD may inhibit thrombin-induced FGF-2 release, presumably via increased cell membrane stiffness.[20] Another mechanism which could be involved in the observed increase in FGF-2 release by thrombin in cholesterol rich cells may be via increased expression of the PARs or by affecting intracellular GPCR signaling. However, expression levels of PAR mRNAs and of PAR1 protein on the cell surface as determined by flow cytometry were not affected by cholesterol (data not shown). Whether cholesterol affects GPCR-dependent intracellular signaling has not been investigated here.

Because cholesterol accumulation did not only neutralize the inhibitory effect of MbCD on thrombin-induced FGF-2 release but even caused an increased release of FGF-2 by thrombin, we investigated whether cholesterol may stimulate FGF-2 expression. An induction of FGF-2 by a different procedure of elevating cellular cholesterol has been described before in rabbit SMCs.[18] In human SMCs, cholesterol loading caused a concentration-dependent induction of FGF-2 mRNA (Figure 3A). Figure 3B shows induction of FGF-2 mRNA determined by real-time PCR. This induction was maximal after 12 hours of cholesterol treatment and was maintained over 24 hours (not shown). Figure 3C and 3D demonstrates increased expression of FGF-2 protein by cholesterol, whereas MbCD had no effect on FGF-2 protein or mRNA levels. In comparison to upregulation of FGF-2, the known receptors involved in FGF-2 signaling—FGFR-1 and syndecan-4[24]—were not affected by cholesterol or MbCD (data not shown).

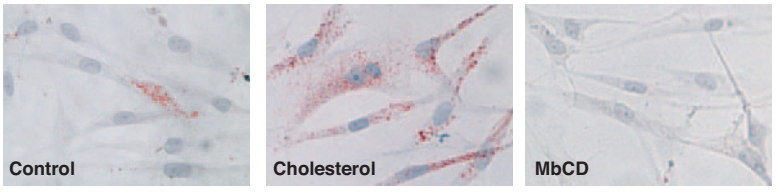

Figure 2. Cholesterol enrichment in human SMCs. Cells were incubated with MbCD-cholesterol (10 μg/mL cholesterol) or with the equivalent amount of MbCD for 24 hours. Cholesterol was stained with oil-red O.

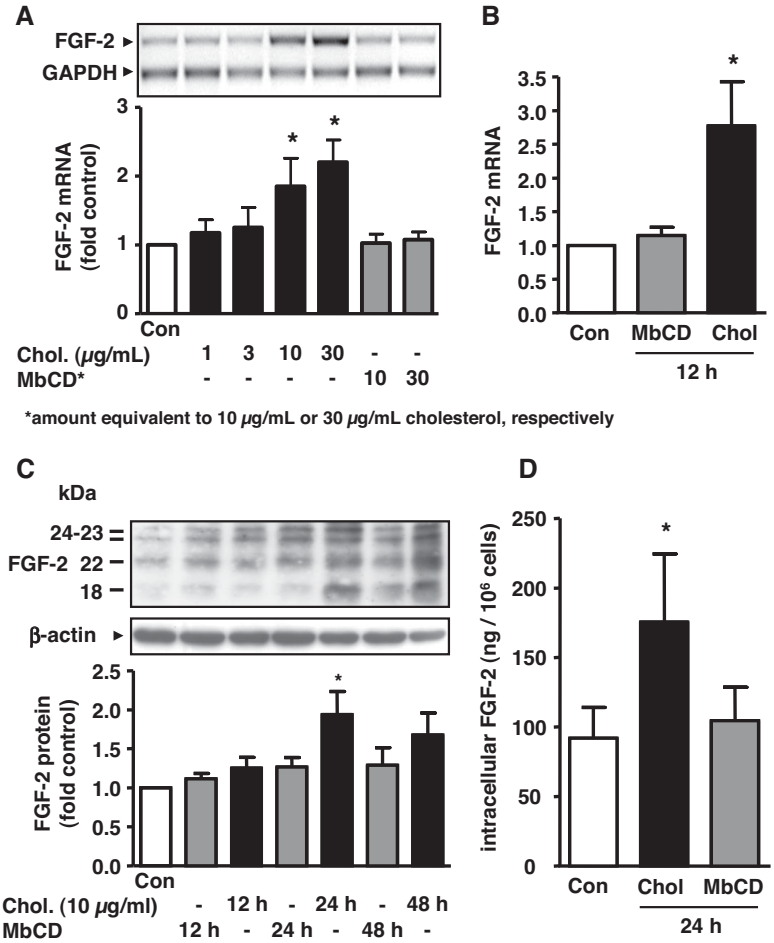

Figure 3. Cholesterol enrichment stimulates FGF-2 expression in human SMCs. Cells were incubated with increasing concentrations of MbCD-cholesterol or with equivalent amounts of MbCD as indicated. FGF-2 mRNA was determined by semi-quantitative RT-PCR with GAPDH as internal control (A) and by real-time PCR (B); FGF-2 protein was determined by Western blotting (C) and by ELISA (D). Four isoforms of FGF-2 ranging from 24.1 to 18 kDa have been described.[31] Values are mean±SEM of 5 (A, B, D) and 7 (C) independent experiments, *$P<0.05$ cholesterol vs controls.

Thus, increased expression of FGF-2 is likely to account for its enhanced release (Figure 1C and 1D) and the enhanced mitogenic response of cholesterol-treated SMCs after stimulation with thrombin as compared with control and MbCD-treated cells (Figure 4). Consistently, neutralizing FGF-2 by specific antibodies inhibited thrombin-induced DNA synthesis (Figure 4A) and cell proliferation (Figure 4B) in cholesterol-enriched cells to a similar level as pretreatment with MbCD. In agreement with previous results,[6,24] neutralizing FGF-2 inhibited thrombin-induced DNA synthesis also in the untreated control group. However, when SMCs were pretreated with MbCD the FGF-2-neutralizing antibody did not yield any additional inhibition on thrombin-mediated DNA synthesis (Figure 4A). Similar results were observed for cell counts (Figure 4B). Whereas MbCD-cholesterol enhanced SMC proliferation induced by thrombin, MbCD and FGF-2-neutralizing antibody inhibited thrombin-induced cell proliferation. In line with these effects of cholesterol loading on thrombin-induced DNA synthesis and proliferation, we observed a pronounced phosphorylation of extracellular signal regulated kinase (ERK) 1/2 in cholesterol-rich SMCs in the late phase of thrombin-induced ERK phosphorylation (Figure 4D). This is consistent with previous observations that the late phase of thrombin-induced ERK phosphorylation is FGF-2 dependent.[24] To determine potential signaling pathways which may be involved in the release of FGF-2 by thrombin, we incubated the cells with various inhibitors of signaling pathways which are either known to be important in thrombin-dependent mitogenesis or are associated with the release of intracellular proteins. Whereas pretreatment with the Rho-kinase inhibitor Y27632 inhibited thrombin-induced release of FGF-2 in untreated and in cholesterol-rich SMCs (Figure 4C), the PKC inhibitor GF109203X, the MAPK inhibitor PD98059, the tyrosine kinase inhibitor genistein, the intracellular calcium chelator BAPTA/AM, EGTA, the cytoskeleton inhibitor colchicin, and the potassium channel inhibitor adenylyl-imidodiphosphate did not reduce thrombin-dependent FGF-2 release (not shown). Y27632 alone had no effect on basal FGF-2 release. In agreement with the literature, incubation with Y27632[28] (1 to 10 μmol/L) or with PD98059[29] (10 to 40 μmol/L) inhibited thrombin-induced DNA synthesis in both, control cells and cholesterol-rich cells (not shown). Taken together, these observations indicate that the Rho/Rho kinase pathway may be involved in thrombin-triggered FGF-2 release, whereas ERK activation—although required for thrombin- and cholesterol-mediated mitogenesis—is not essential for the release of FGF-2. However, further investigation will have to follow to clarify this pathway in more detail. In addition, MbCD has been shown to affect FGF-2 signaling probably by disruption of lipid rafts.[21] Lipid rafts are cholesterol-rich membrane domains where FGF-2–binding HSPG like syndecan-4 clus-

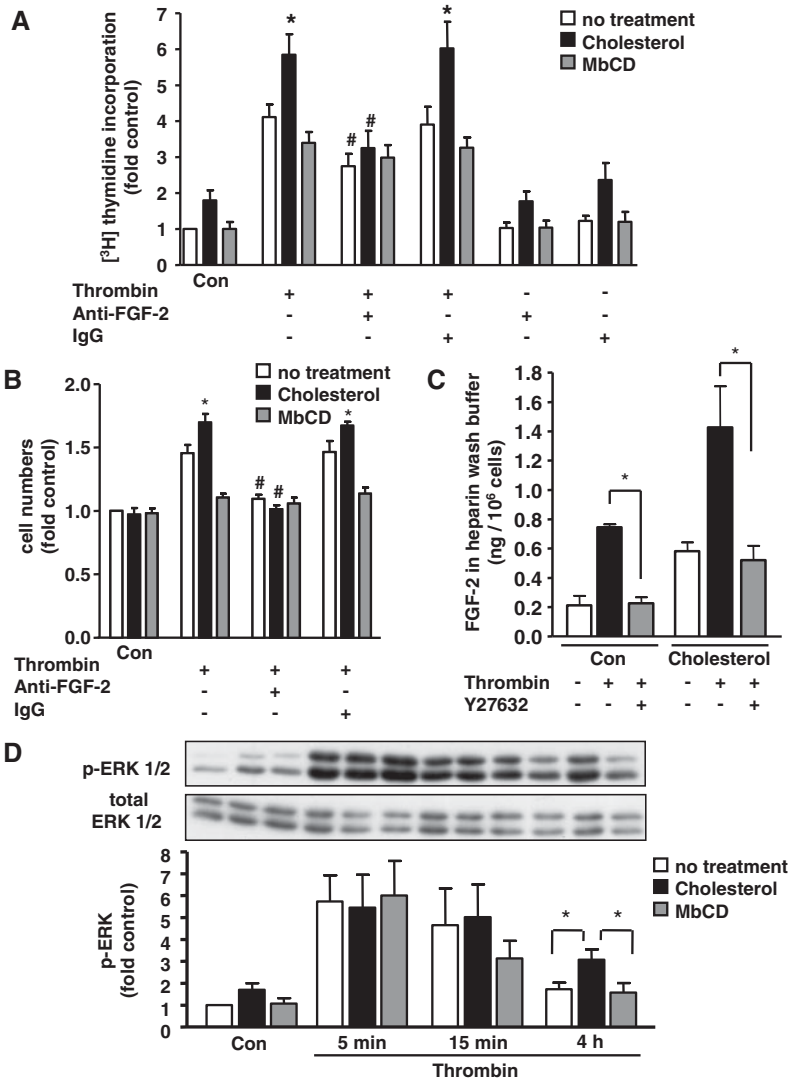

Figure 4. Thrombin-induced mitogenesis is enhanced by cholesterol enrichment. SMCs were either untreated or pretreated with MbCD-cholesterol or MbCD for 24 hours. DNA synthesis was determined by thymidine incorporation after incubation with thrombin (30 nmol/L) for 24 hours in the absence or presence of FGF-2–neutralizing antibody (anti-FGF-2, 30 μg/mL) or control IgG (30 μg/mL) (A). Proliferation was determined by cell counts after incubation with thrombin for four days (B). Values are mean±SEM of 8 (A) or 6 (B) independent experiments performed in triplicate, $^{*}P<0.05$ for cholesterol vs no treatment and MbCD, $^{\#}P<0.05$ for anti–FGF-2 pretreatment vs thrombin. Release of FGF-2 into the pericellular matrix from untreated (Con) and cholesterol-rich SMCs was determined by ELISA (C) after preincubation with the Rho-kinase inhibitor Y27632 (1 μmol/L) for 1 hour followed by incubation with thrombin (30 nmol/L) for 10 minutes (mean±SEM of 4 independent experiments, $^{*}P<0.05$ as indicated). Phosphorylation of ERK 1/2 (p-ERK) in control cells, MbCD-cholesterol-, or MbCD-treated cells after incubation with thrombin (30 nmol/L) for the indicated times was determined by phospho-specific antibodies on Western blots (D). Total ERK is shown as control. Density values are mean±SEM of 6 independent experiments, $^{*}P<0.05$ as indicated.

ter.[30] Whether disruption of lipid rafts contributes to the MbCD effect on thrombin-induced mitogenesis or may be involved in the release of FGF-2, remains to be determined.

In summary, the present study demonstrates that thrombin-induced mitogenesis is enhanced at elevated intracellular cholesterol levels in human vascular SMCs. This might be attributable to enhanced release of FGF-2 by thrombin, presumably via cholesterol-induced transcriptional upregulation of FGF-2 gene expression. This suggests that cellular cholesterol levels may indirectly control thrombin-dependent mitogenesis in human SMCs by modulating FGF-2 expression.

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Disclosures

None.

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Arbeit #8

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*Pape R, *Rauch BH, Rosenkranz AC, Kaber G, Schrör K.

Prostacyclin inhibits expression of protease-activated receptor-1 PKA-dependently in human vascular smooth muscle cells.

(In Revision bei *Arterioscler Thromb Vasc Biol.*)

**Beide Autoren trugen zu gleichen Teilen zum Manuskript bei.*

Prostacyclin Inhibits the Response to Thrombin via Transcriptional Regulation of Protease-Activated Receptor-1 in Human Vascular Smooth Muscle Cells

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Abstract

Activation of protease-activated receptor-1 (PAR-1) by thrombin stimulates vascular smooth muscle cell (SMC) mitogenesis and has been implicated in the response to vascular injury. Vessel injury is also associated with enhanced formation of vasodilatory prostaglandins (PG). We investigated whether PGI_2 (prostacyclin) and PGE_2 affect expression of PAR-1 and the cellular response to thrombin in human SMC. PGE_2 inhibited PAR-1 expression only at supra physiological concentrations. The PGI_2-mimetic iloprost (1 - 100 nmol/L) attenuated mRNA, total protein and cell surface expression of PAR-1. Accordingly, iloprost inhibited thrombin-induced DNA synthesis. Comparable inhibition of PAR-1 expression was observed with the selective IP-receptor agonist cicaprost, the adenylyl cyclase activator forskolin, the phosphodiesterase inhibitor isobutylmethylxanthine and the PKA activator dibutyryl-cAMP. The specific PKA-inhibitor Myr-PKI prevented PAR-1 downregulation by iloprost. The potential role of Rho family GTPases in PAR-1 regulation was also investigated. Iloprost decreased Rac1 mRNA and the Rac1 inhibitor NSC23766 mimicked the inhibitory effects of iloprost on PAR-1 protein- but not mRNA expression. The Rho kinase inhibitor Y27632 had no effect on PAR-1 expression. In conclusion, IP-receptor ligands may limit the mitogenic actions of thrombin in human SMC by downregulating PAR-1 via modulation of cAMP-/PKA- and Rac1-dependent signaling pathways.

Keywords

protease-activated receptor-1, smooth muscle, thrombin, prostaglandins, PKA

Introduktion

The serine protease thrombin is generated at sites of vessel injury and is a key factor in blood coagulation. In addition, thrombin exerts direct effects on vascular smooth muscle cells (SMC), including cell proliferation and migration which may contribute to vascular lesion formation.[1] The vascular actions of thrombin are mediated via activation of G-protein coupled protease-activated receptors PAR-1, PAR-3 and PAR-4.[2,3] A further receptor, PAR-2, is activated by other proteases, including trypsin and factor Xa.[4] Activation of PARs occurs by proteolytic cleavage of their extracellular domain. This unmasks a new N-terminus which functions as a tethered peptide ligand[4] and triggers G-protein binding and intracellular signaling. Synthetic peptides containing the sequence of the tethered ligand selectively activate their respective receptor (PAR-activating peptides, PAR-AP) and mimic activation by thrombin.[5] Upon stimulation, PARs are internalized by phosphorylation-dependent mechanisms and subsequently delivered to lysosomes for degradation.[6] Reappearance of uncleaved receptors at the cell surface requires de novo synthesis or delivery from intracellular stores.[7,8]

PAR-1 represents the prototypic thrombin receptor. Targeted disruption of the PAR-1 gene in mice has demonstrated its relevance for embryonic development and cellular responsiveness to thrombin.[9] In addition, a role of PAR-1 has been implicated in the development and progression of cardiovascular disease,[10,11] particularly in neointima formation after vascular injury.[12,13]

Vessel injury is associated with induction of cyclooxygenase-2 (COX-2), causing enhanced formation of vasodilatory prostaglandins (PG) at sites of atherosclerotic or restenotic lesions.[14-16] Under these conditions, SMC themselves are an important source of PG synthesis,[14] predominantly PGE_2 and PGI_2 (prostacyclin).[17] PGI_2 exerts important atheroprotective actions[18] including inhibition of platelet adhesion and aggregation,[19] and inhibition of SMC proliferation[20,21] and migration.[22] Such functional antagonism by PGI_2 involves in part suppression of thrombin-induced expression of the growth regulatory gene cyr61[23] and upregulation of thrombomodulin, an important inhibitor of blood coagulation.[24] Whether vasodilatory PGs such as PGI_2 or PGE_2 may also regulate thrombin actions by modulating PAR expression in vascular SMC has not yet been investigated. Because cells surface expression of PAR-1 has recently been reported to depend on the Rho family member Rac1[25], we determined whether small GTPases[26] may be involved in the potential prostacyclin-dependent regulation of PAR-1 expression.

The present study reports that the PGI_2 analog iloprost attenuates mRNA, total protein and surface expression of PAR-1. This is associated with a reduced mitogenic response to thrombin. Gs-/cAMP-/PKA- and Rac1-dependent signaling pathways appear crucial for the inhibitory effect of iloprost on PAR-1 expression. Taken together, we provide first evidence that vasodilatory PG control the mitogenic response of thrombin at the level of PAR expression in human SMC.

Material and Methods

Materials

Dulbecco´s modified eagle medium (DMEM) and fetal calf serum (FCS) were from GIBCO BRL (Rockville, MD, USA). Purified α-thrombin was kindly provided by Dr. J. Stürzebecher (Institut für Vaskuläre Medizin, Jena, Germany); iloprost and cicaprost by Schering AG (Berlin, Germany). PGE_2 was obtained from Cayman Chemical Company (Ann Arbor, MI, USA); 3-isobutyl-1-methylxanthine (IBMX), forskolin and dibutyryl cAMP (db-cAMP) were from Sigma-Aldrich (Taufkirchen, Germany). Myristoylated protein kinase A inhibitor (Myr-PKI), inhibitors of Rac1 (NSC23766) and Rho-kinase (Y-27632) were from Calbiochem (San Diego, CA, USA). Gene-specific primers were from Invitrogen (Karlsruhe, Germany). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human SMC were isolated from saphenous veins by the explant technique and cultured as described previously.[27] For experiments, subconfluent cells at passages 4-10 were serum-deprived for 24 hours prior to treatment with the different substances for the indicated times.

Flow cytometry

For analysis of surface expression of PAR-1, SMC were seeded in 6-well plates and stimulated as indicated. After non-enzymatic detachment with citric saline buffer (0.135 mol/L potassium chloride, 0.015 mol/L sodium citrate) for 10 - 15 min at 37°C, cells were pelleted and resuspended in PBS. Cell suspensions (50 µl) were incubated with 10 µl PE-conjugated anti-human PAR-1 antibodies (Coulter-Immunotech, Marseille Cedex, France) for 20 min at room temperature in the dark. Isotype-matched PE-conjugated antibodies were used to assess non-specific binding. Samples were diluted with 500 µl Isotone® and immediately analysed on an EPIC-XL cytometer (Beckman Coulter, Krefeld, Germany). SMC populations were identified according to forward and side scatter distributions. Detectors were set to logarithmic amplification and fluorescence was measured in 7,500 cells using the System II (3.0) software. For quantification, the ratios of the mean fluorescence signals of PAR-1- and non-specific IgG_1-stained cells were normalized to the unstimulated control.

Immunocytochemistry

SMC plated on 8-well chamber slides (LabTek; Nunc) at a density of 10,000 cells/cm^2 were incubated as indicated, then fixed for 20 min (freshly made 3.7% paraformaldehyde) and blocked for 1 hour (3% BSA in PBS). Cells were then incubated with anti-PAR-1 antibodies (ATAP2; Santa Cruz Biotechnology; 1:50 in 1% BSA/PBS) overnight followed by incubation with HRP-conjugated secondary antibodies (goat anti-mouse; Santa Cruz Biotechnology; 1:400 in 1% BSA/PBS) for 1 hour. Diaminobenzidine was used as chromogen. Nuclei were stained with

hemalaun. Images were taken with a colorview II camera and SIS software (Soft Imaging System) connected to an Olympus BX 50 microscope.

Semi-quantitative RT-PCR and real-time PCR

Total RNA from SMC was prepared with TriFast reagent (peqLab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions. RT-PCR was performed with a One-Step RT-PCR kit (Qiagen, Hilden, Germany) using 250 ng RNA. Genespecific primers (0.6 µmol/L each) were: PAR-1 sense CCA CGG CAG ATG TGC TGT TTG, antisense TAG GCA GCC TCT GTG GTG GAA G; Rac1 sense CCC TAT CCT ATC CGC AAA CA, antisense CAG CAG GCA TTT TCT CTT CC. GAPDH primers were used as described.[28] Thermal cycler conditions were: 1 min 94°C, 1 min annealing (58°C for PAR-1, 62°C for Rac1), 1 min 72°C and elongation at 72°C. After separation in agarose gels, PCR fragments were visualized and quantified on a Biorad GelDoc instrument. For real-time PCR, total RNA was reverse-transcribed into cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems). PAR-1 mRNA expression was determined using SYBR®Green Master Mix (Applied Biosystems) and QuantiTect Primer Assay (Qiagen, Hilden, Germany) QT00230489 (PAR-1) and QT00199367 (ribosomal 18S as internal control) according to the manufacturer's instructions. PCR was performed on a 7300 Real Time PCR System (Applied Biosystems). PAR-1 expression levels relative to 18S were determined using the $\Delta\Delta$Ct method[29] and expressed relative to paired controls.

Western Blot Analysis

PAR-1 expression was detected in whole cell lysates by Western blotting using monoclonal anti-PAR-1-antibodies (ATAP2, Santa Cruz Biotechnology). After treatment with the indicated agents, cell lysates were resolved by SDS polyacrylamide gel electrophoresis as described previously.[27] Bands were visualised by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK).

DNA synthesis

DNA synthesis was determined by [^{3}H] thymidine incorporation as described previously.[27,28]

Statistical analysis

Data are means $\pm$ S.E.M. from n experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post-hoc Bonferroni multiple comparisons procedure. $P < 0.05$ was considered as significant.

Results

Effects of iloprost and PGE_2 on PAR-1 mRNA and total protein expression

Levels of PAR-1 mRNA in cultured human SMC were determined by semi-quantitative RT-PCR and quantitative real-time PCR. Incubation of cells with the stable PGI_2-mimetic iloprost (100 nmol/L) time-dependently reduced expression of PAR-1 mRNA within 6 to 24 hours (figure 1A). Nanomolar concentrations of iloprost (10 – 100 nmol/L) were sufficient to inhibit PAR-1 mRNA expression (figure 1B, equivalent data obtained by semiqantitative RT-PCR not shown). Incubation with iloprost also strongly attenuated total amount of PAR-1 protein as demonstrated by Western blotting (figure 1E). Molecular identity of PAR-1 was indicated by size as well as by a shift in mobility of the receptor band in SDS polyacrylamide gel electrophoresis after treatment of cells with thrombin, indicating proteolytic cleavage of the N-terminus (not shown). PGE_2 also inhibited PAR-1 mRNA (figure 1C and 1D) and total protein expression (figure 1F) however only at micromolar (1 – 10 µmol/L) but not at nanomolar concentrations (1 – 100 nmol/L, not shown).

Inhibition of PAR-1 surface expression by iloprost and PGE_2

Changes in PAR-1 surface expression were assessed by flow cytometry. Incubation of cells with iloprost for 24 hours (1 - 100 nmol/L) resulted in decreased PAR-1 cell surface expression as indicated by reduced fluorescence intensity (figure 2A). Fluorescence-labeled isotype-matched IgG were used as control. Quantitative analysis revealed 40 – 50 % suppression by the indicated iloprost concentrations (Figure 2A). Treatment of cells with PGE_2 inhibited cell surface expression of PAR-1 at high concentrations (1 – 10 µmol/L, figure 2B), while lower concentrations (1 - 100 nmol/L) had no effect on PAR-1 surface expression (not shown). Moreover, immunocytochemistry experiments using non-permeabilized SMC were performed. In control cells, a strong immunoreactivity indicated the presence of PAR-1 at the cell surface (figure 2C), which was markedly reduced after incubation with iloprost (10 nmol/L) for 24 hours.

Involvement of G_s/cAMP/PKA-dependent signaling pathways in the iloprost-mediated regulation of PAR-1 expression

PGI_2 has been shown to activate stimulatory G-protein (G_s)-coupled IP-receptors leading to intracellular cAMP formation and activation of protein kinase A (PKA).[18] Treatment of human SMC with either the selective IP-receptor agonist cicaprost (1 - 10 nmol/L) or the adenylyl cyclase activator forskolin suppressed PAR-1 mRNA and cell surface protein expression (figure 3). Treatment with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mmol/L) or the membrane-permeable PKA-activator dibutyryl-cAMP (db-cAMP, 1 mmol/L) mimicked the effects of iloprost on PAR-1 mRNA expression (figure 3D). Incubation with the highly specific PKA inhibitor Myr-PKI (5 µmol/L) prior to addition of iloprost prevented downregulation of PAR-1 mRNA, total protein and cell surface expression (figure 4), confirming the significance of PKA signaling in iloprost-mediated regulation of PAR-1 expression in human SMC.

Downregulation of PAR-1 attenuates the mitogenic response to thrombin

To assess whether the PG-mediated decrease of PAR-1 surface expression is associated with attenuated PAR-dependent mitogenesis, we determined DNA synthesis by [^{3}H]-thymidine incorporation in response to thrombin or a selective PAR-1-activating peptide (PAR-1-AP). Figure 5A demonstrates that in control cells, both thrombin (3 U/ml) and PAR-1-AP (TFLLRN, 200 µmol/L) induced approximately 3.5- and 2-fold increases in DNA synthesis, respectively. Pretreatment with iloprost for 24 hours significantly reduced the mitogenic response to thrombin and PAR-1-AP, while preincubation for 1 hour was ineffective. In contrast, preincubation with iloprost had no effect on DNA synthesis induced by PDGF-BB (10 ng/ml) or FCS (10%) (figure 5B).

Role of Rho family GTPases for the regulation of PAR-1 expression by iloprost

The GTPase Rac1, a member of the Rho family of small G-proteins, has recently been suggested to play a crucial role in maintaining surface expression of PAR-1.[25] Therefore, we investigated a possible role of Rac- and Rho-dependent signaling in iloprost-mediated regulation of PAR-1 expression. Iloprost significantly attenuated Rac1 mRNA within 24 hours (figure 6A). Selective inhibition of Rac1 activity with the cell-permeable pyrimidine compound NSC23766 (50 µmol/L)[30] time-dependently reduced PAR-1 surface expression (figure 6B and 6D) as well as total PAR-1 protein in the cells (figure 6E). In contrast, inhibition of Rho-associated protein kinase (ROCK) with Y27632 (10 µmol/L)[31] did not affect PAR-1 surface expression (figure 6C and 6D) or total PAR-1 protein (figure 6E). None of these inhibitors altered PAR-1 mRNA expression (figure 6E). Thus, modification of Rac1- but not Rho-dependent signaling appears to be involved in the regulation of PAR-1 surface expression by iloprost in human SMC.

Discussion

PARs are the major targets of coagulation factor signaling on vascular cells.[3] They play a crucial role in cardiovascular physiology and have been implicated in atherosclerosis and inflammation.[11] Therefore, understanding the mechanisms that modulate PAR expression is relevant for also understanding such pathological processes. This study has examined the regulation of PAR-1 expression by the PGI_2-mimetic iloprost. We reveal that vasoactive PGs inhibit the mitogenic actions of thrombin by down-regulating PAR-1 in human SMC.

Expression of PAR-1 was determined at the level of mRNA, total protein and cell surface localization (figure 1 and 2). We found that long-term exposure (24 hours) to iloprost inhibited surface expression of PAR-1 as demonstrated by flow cytometry (figure 2). Since suppression of PAR-1 protein and mRNA developed rather slowly, decreased PAR-1 *de novo* synthesis is likely to account for the observed reduction of PAR-1 levels at the cell surface. In agreement with this hypothesis, the inhibitory effect of iloprost on DNA synthesis induced by thrombin or PAR-1-AP were observed after 24 hours preincubation with iloprost, while short-term exposure (1 hour) was ineffective (figure 5). Whether additional mechanisms such as increased internalization and degradation contribute to the reduction of total PAR-1 protein by PG needs to be elucidated in future studies. Incubation with cycloheximide did not prevent downregulation of PAR-1 indicating that *de novo* protein synthesis is not required for this effect (not shown).

The intracellular signal transduction pathways involved in regulation of PAR-1 expression were investigated. We postulate that the prostanoid-evoked inhibition of PAR-1 expression is mediated primarily via an increase in intracellular cAMP formation and subsequent PKA activation, as it was mimicked by cAMP-elevating agents like forskolin, isobutylmethylxanthine or the membrane-permeable cAMP-analogue dibutyryl-cAMP (figure 3). In addition, iloprost-mediated inhibition of PAR-1 expression was attenuated by the cell permeable specific PKA inhibitor Myr-PKI[32] (figure 4). Similar data were obtained with the selective PKA inhibitor adenosine 3′,5′-cyclic phosphorothioate-Rp isomer (Rp-cAMPs, not shown). These observations are in agreement with reports describing cAMP-dependent downregulation of PAR-1 in different human cell systems including lung fibroblast and mesangial cells,[33,34] and with another recent finding that iloprost activates PKA leading to differentiation of SMC.[32] Moreover, the selective IP receptor agonist cicaprost mimicked the effects of iloprost in our study, suggesting that stimulation of Gs-coupled IP receptors causes inhibition of PAR-1 expression.

While nanomolar concentrations of iloprost significantly inhibited PAR-1 expression, considerably higher PGE_2 concentrations (10 µmol/L) were required to achieve equivalent inhibition. A possible explanation for this differential effect is provided by our recent observation that PGE_2 is far less potent in increasing intracellular cAMP levels than iloprost in human SMC,[35] which may be due to simultaneous activation of cAMP-lowering Gi-coupled EP3 receptors.[36] Thus, it is likely that PGI_2 rather than PGE_2 is the major prostanoid responsible for modulating thrombin effects *in vivo*. Nevertheless, synergistic effects of both prostanoids may result in a pronounced inhibition of PAR-1 in vascular SMC at sites of COX-2 induction, such as in the vicinity of vascular lesions.[16]

To further characterize the signaling pathways mediating expression of PAR-1 by PGI_2, we examined the role of the Rho-GTPases Rac1 and Rho, which have been suggested to play an important role in regulation of receptor endocytosis.[37,38] The use of the selective Rac1 inhibitor NSC23766 revealed that Rac-dependent pathways are involved in regulating PAR-1 cell surface expression in human SMC (figure 6). PAR-1 mRNA expression was however not affected by the inhibitor indicating that Rac1 controls surface protein expression of PAR-1 via regulation of constitutive trafficking (e.g. internalization and degradation of receptor protein) and not via transcriptional changes. Our data are in agreement with observations that constitutive surface expression of PAR-1 in vascular SMC is controlled by Rac1.[25] In contrast, the Rho-associated protein kinase inhibitor Y-27632 did not affect expression of PAR-1, suggesting that Rho-dependent signaling is not involved in trafficking of this receptor. To rule out the possibility that Y-27632 concentrations applied were not sufficient to inhibit Rho-dependent signaling we assessed thrombin-induced DNA synthesis, which is known to depend on activation of Rho.[39] Y-27632 at similar concentrations (1 - 10 µmol/L) effectively inhibited thrombin-induced DNA synthesis (not shown).

In conclusion, we report for the first time that PAR-1 expression is inhibited by vasodilatory PG in human vascular SMC. This suggests that in atherosclerotic or restenotic vessels, induction of PG synthesis via the COX-pathway in medial or neointimal SMC, as well as long-term exposure of SMC to PG from other sources such as macrophages, might result in reduction of cell responsiveness to PAR activation. This mechanism might serve to counteract local mitogenic and other cellular actions of thrombin as well as of additional proteases that are generated at sites of vascular injury, such as activated coagulation factor-X, and have also been described to cleave PAR-1.[40]

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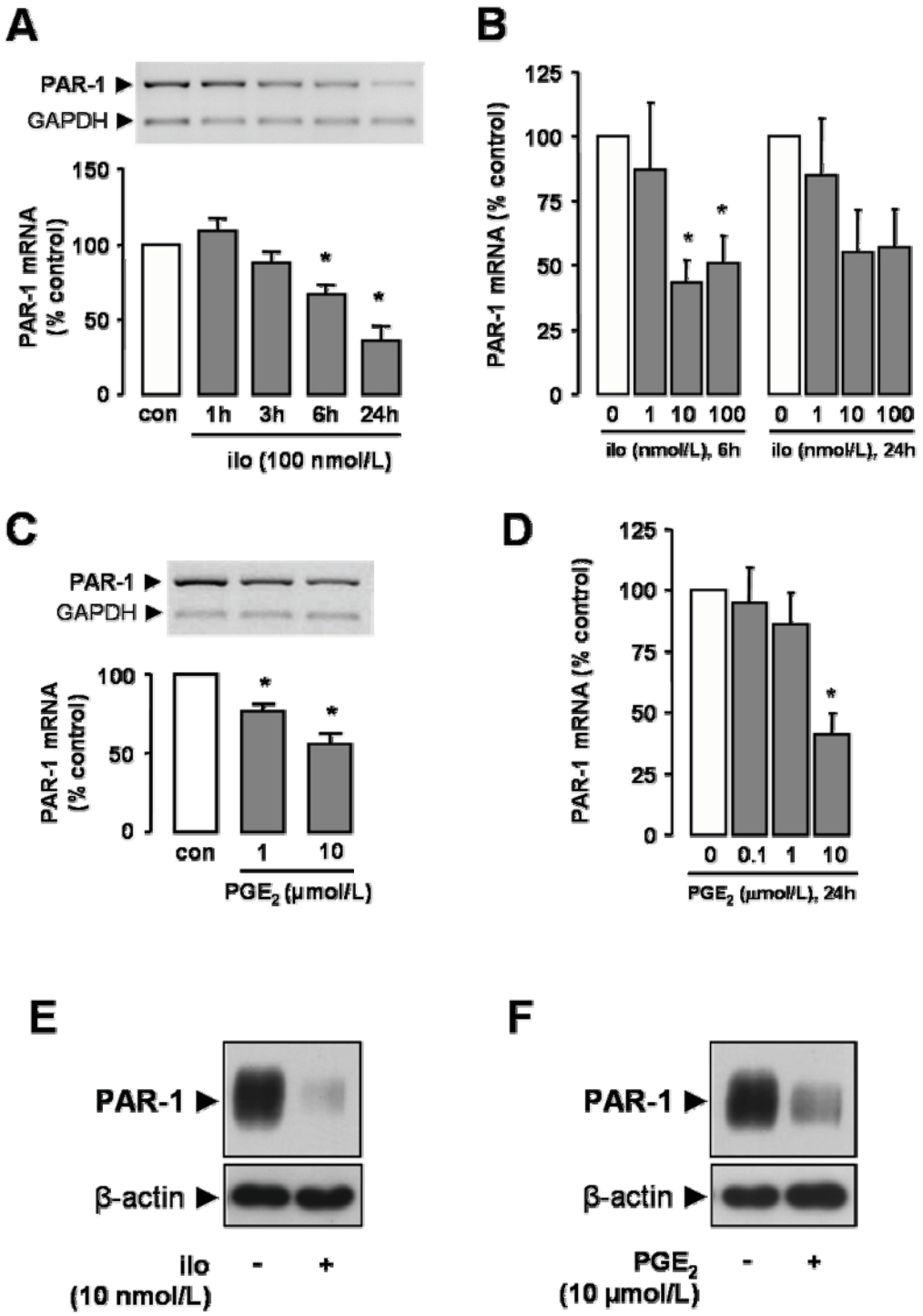

Figure 1

Inhibition of PAR-1 mRNA and total protein expression in human SMC. A, PAR-1 mRNA levels were determined by semiquantitative RT-PCR after incubation with iloprost for the indicated times. **B**, Determination of PAR-1 mRNA by real-time PCR after incubation with increasing concentrations of iloprost for 6 or 24 hours. **C and D**, PAR-1 mRNA expression after incubation with PGE_2 for 24 hours. **E and F**, Immunoblot detection of PAR-1 in total lysates of cells treated with or without iloprost or PGE_2 for 24 hours. Blots were reprobed for β-actin to confirm equal protein loading. Values are mean ± SEM of 3-5 independent experiments, *$p < 0.05$ vs. con.

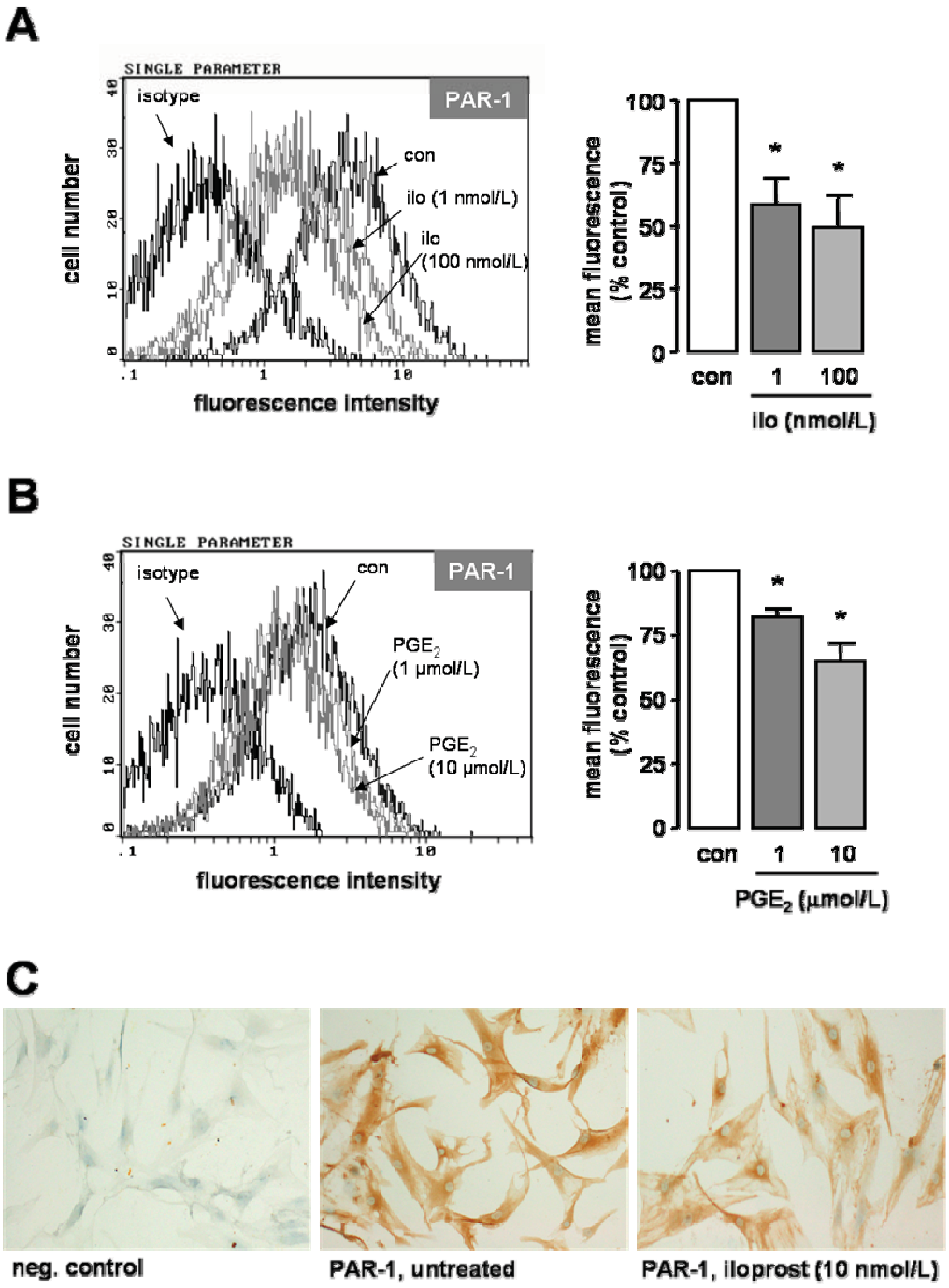

Figure 2

Inhibition of PAR-1 surface expression. Serum-deprived cells were treated with iloprost **(A)** or PGE_2 **(B)** for 24 hours at the indicated concentrations. Representative histograms are depicted on the left. Right panel shows quantitative analysis of the relative mean fluorescence intensities. Values are mean ± SEM of 3 independent experiments, *p < 0.05 vs. con. **C**, PAR-1 expression was determined by immunolabeling with monoclonal anti-PAR-1 antibodies (ATAP2) in untreated cells and after incubation with iloprost (10 nmol/L) for 24 hours. No staining was observed in absence of primary antibody (negative control) as compared to PAR-1 staining (brown). Nuclei were stained with hemalaun (blue). Original magnification: x100. Images are representative of 3 independent experiments.

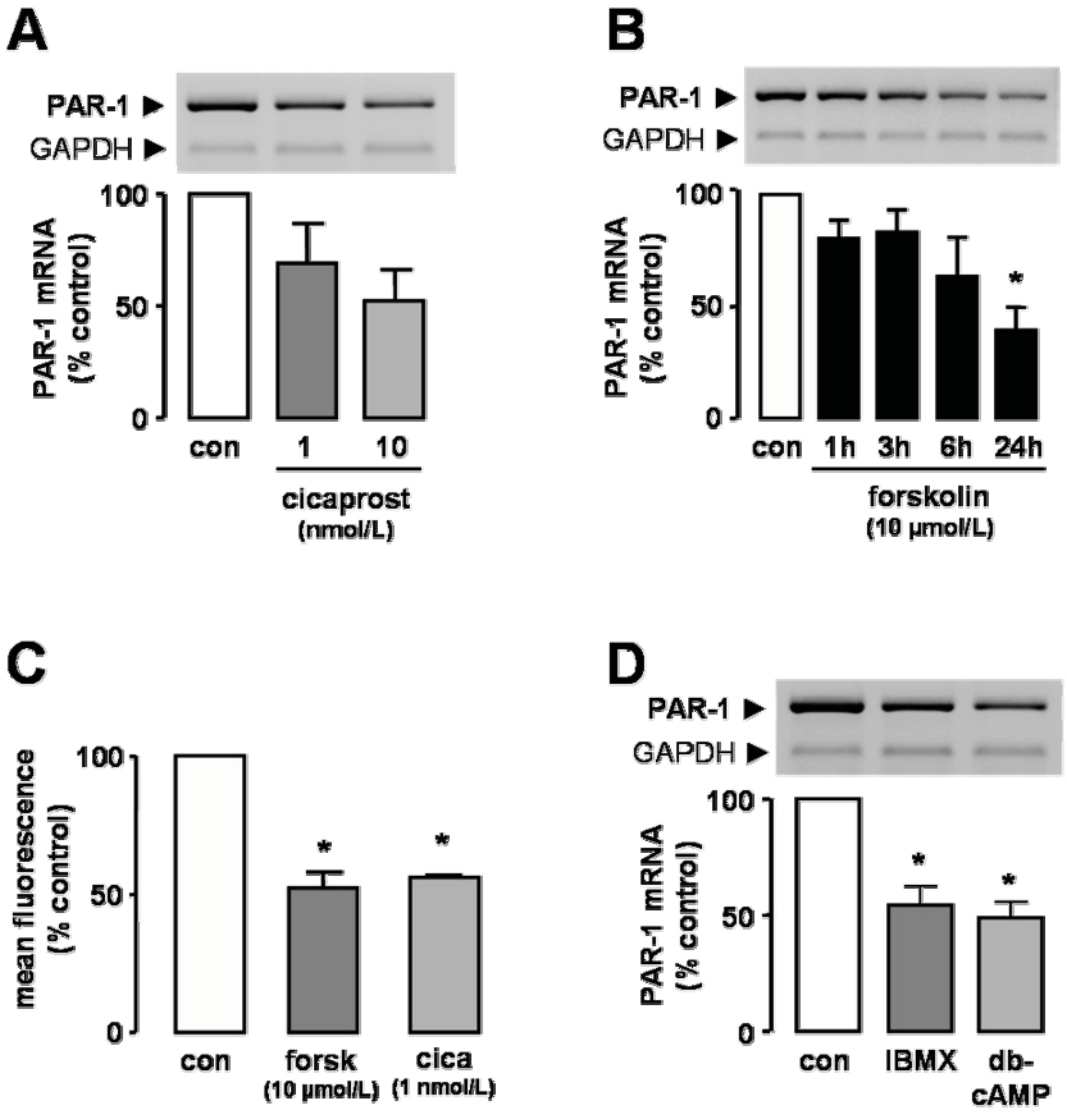

Figure 3

Regulation of PAR-1 expression by iloprost involves Gs-/cAMP-/PKA-dependent signaling pathways. A, PAR-1 mRNA expression after treatment of human SMC with cicaprost (1 - 10 nmol/L) for 24 hours. **B**, Time-dependent downregulation of PAR-1 mRNA by forskolin (10 µmol/L). **C**, Flow cytometry analysis of PAR-1 surface expression in SMC treated with forskolin (forsk, 10 µmol/L) or cicaprost (cica, 1 nmol/L) for 24 hours. **D**, Inhibition of PAR-1 mRNA expression by isobutylmethylxanthine (IBMX, 0.5 mmol/L) and dibutyryl-cAMP (db-cAMP, 1 mmol/L) after 24 hours. In A, B and D, representative RT-PCR experiments and the densitometric quantification (n=3) are shown. Bar graph in C shows quantitative analysis of the relative mean fluorescence intensity measured in at least 3 independent experiments. Values are mean ± SEM, *$p < 0.05$ vs. con.

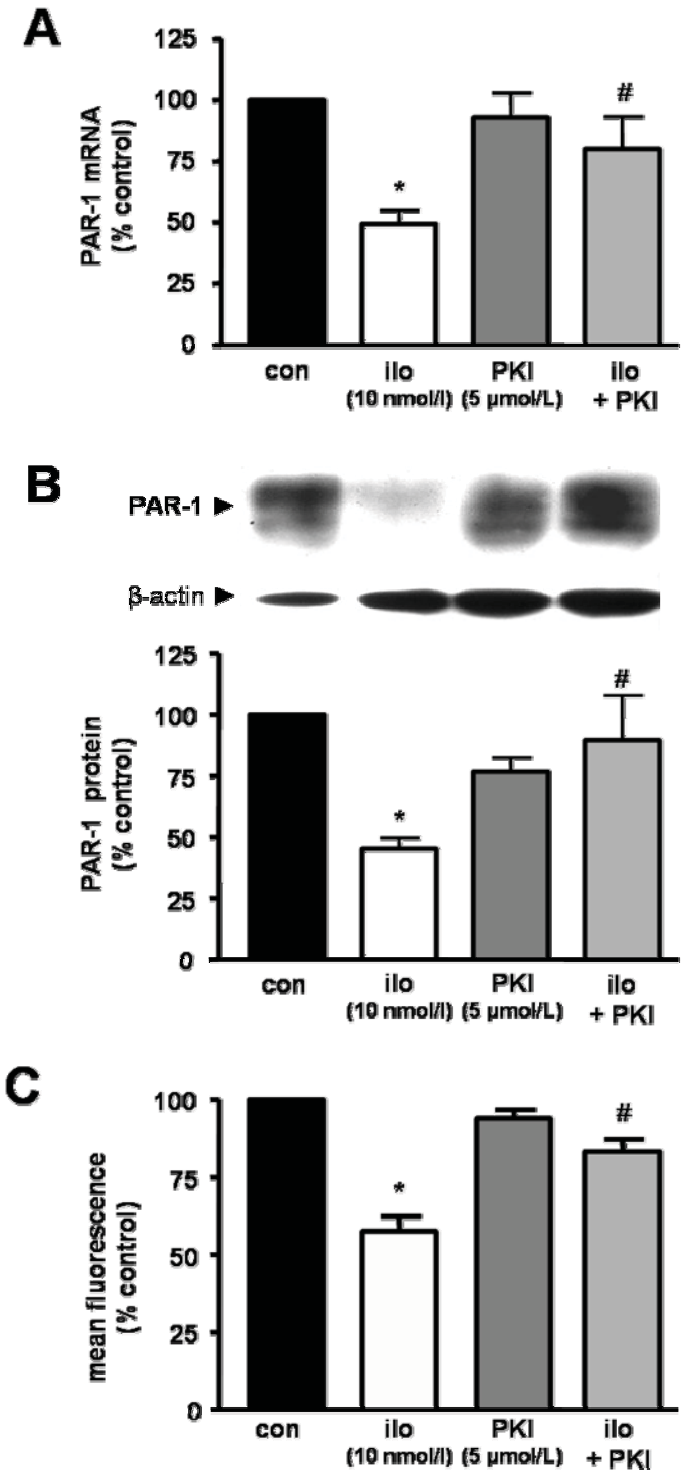

Figure 4

Downregulation of PAR-1 by iloprost depends on PKA activation. SMC were incubated with the cell permeable highly specific PKA inhibitor Myr-PKI (PKI, 5 µmol/L) for 30 minutes prior to incubation with iloprost (10 nmol/L) for 24 hours. Iloprost-induced downregulation of PAR-1 mRNA, total protein, and cell surface expression was inhibited by Myr-PKI as determined by real-time PCR (**A**, n=5), Western blotting (**B**, n=7), and flow cytometry (**C**, n=9), respectively. Values are mean ± SEM, *$p < 0.05$ vs. con, $^{\#}p < 0.05$ vs. ilo.

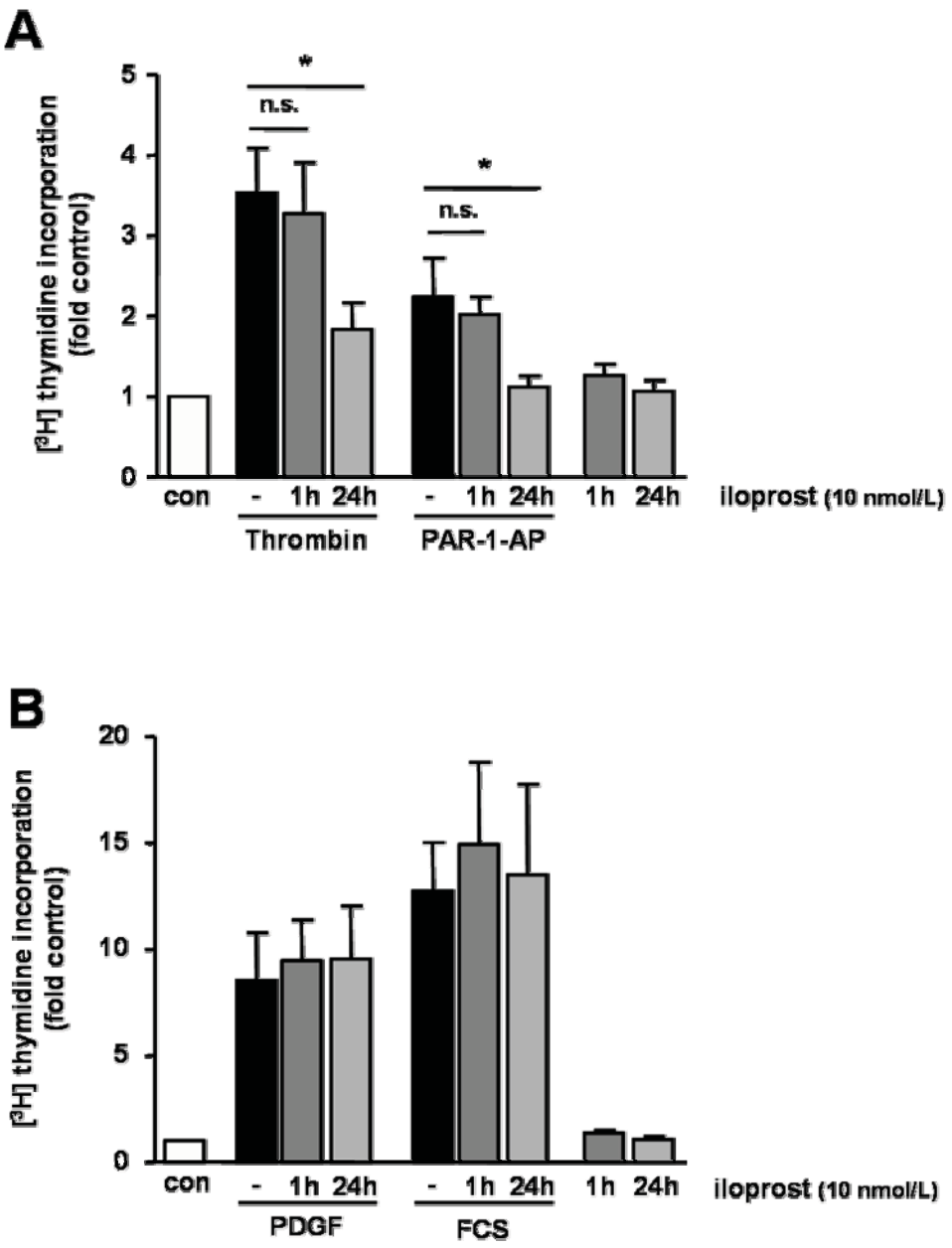

Figure 5

Downregulation of PAR-1 by iloprost attenuates mitogenic response to thrombin. DNA synthesis was determined by [^{3}H]-thymidine incorporation. Where indicated, cells were pretreated with iloprost (ilo, 10 nmol/L) 1 hour or 24 hours before stimulation with (**A**) thrombin (3 U/ml) or PAR-1-actvating peptide (PAR1-AP, 200 µmol/L) or with (**B**) PDGF-BB (10 ng/ml) or FCS (10%). Values are mean ± SEM of 7 independent experiments, *$p < 0.05$ as indicated, n.s. (not significant).

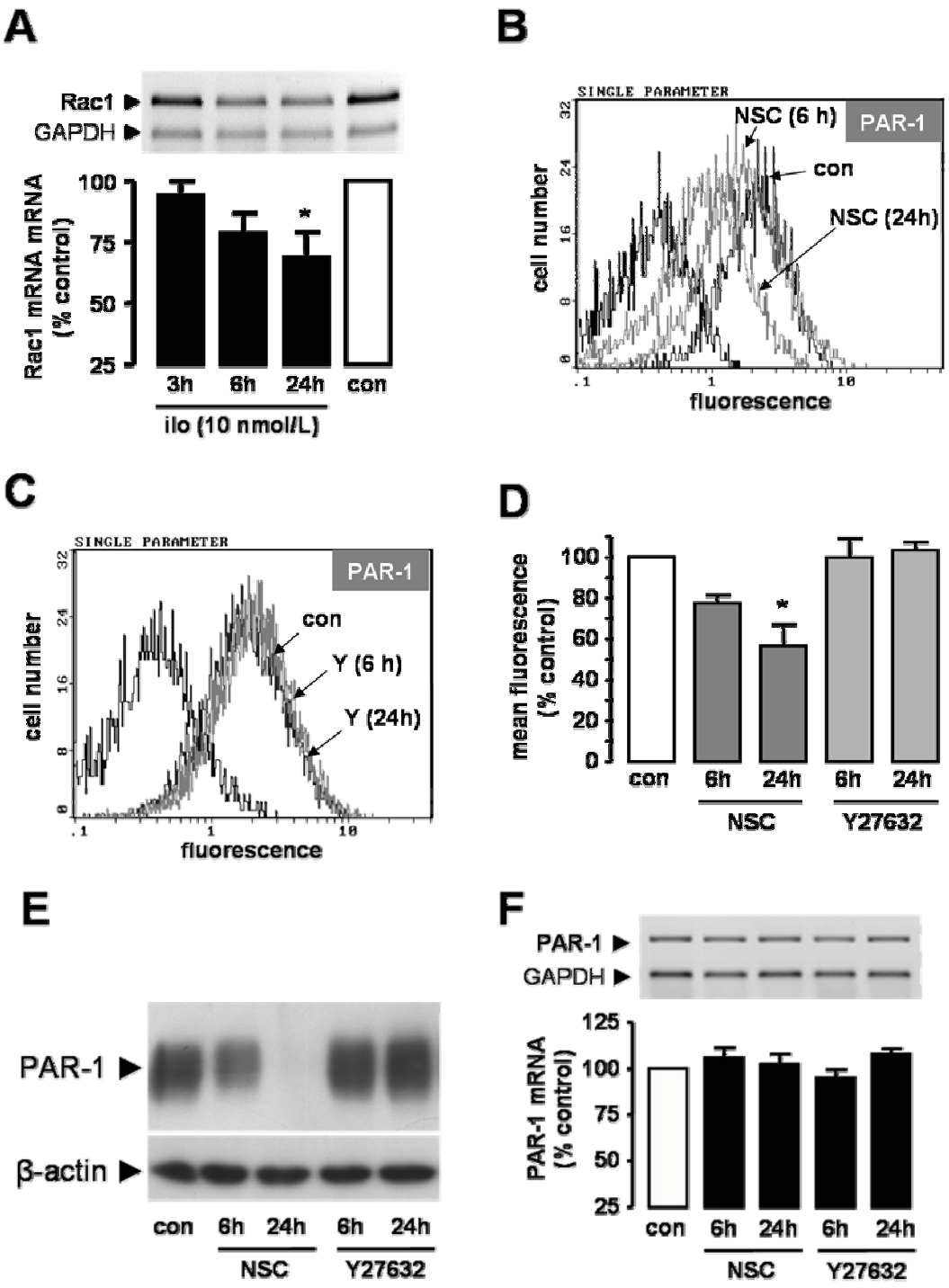

Figure 6

Rac- but not Rho-dependent signaling pathways are involved in regulation of PAR-1 surface expression. Expression of Rac-1 mRNA was determined after incubation with iloprost (10 nmol/L) for the indicated times (**A**). Serum-starved cells were treated with the Rac1 inhibitor NSC23766 (NSC, 50 µmol/L) or the Rho kinase inhibitor Y-27632 (Y, 10 µmol/L) for the indicated times. PAR-1 expression was analyzed by flow cytometry (**B**-**D**), Western blotting (**E**) and RT-PCR (**F**). Values are mean ± SEM of at least 3 independent experiments, *p < 0.05 vs. con.

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