Urokinase-induced signaling in human vascular smooth muscle cells is mediated by PDGFR- β

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Urokinase (uPA)-induced signaling in human vascular smooth muscle cells (VSMC) elicits important cellular functional responses, such as cell migration and proliferation. However, how intracellular signaling is linked to glycolipid-anchored uPA receptor (uPAR) is unknown. We provide evidence that uPAR activation by uPA induces its association with platelet-derived growth factor receptor (PDGFR)- β . The interaction results in PDGF-independent PDGFR- β activation by phosphorylation of cytoplasmic tyrosine kinase domains and receptor dimerization. Association of the receptors as well as the tyrosine kinase activity of PDGFR- β are decisive in mediating uPA-induced downstream signaling that regulates VSMC migration and proliferation. These findings provide a molecular basis for mechanisms VSMC use to induce uPAR- and PDGFR-directed signaling. The processes may be relevant to VSMC function and vascular remodeling.

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Introduction

The urokinase (uPA)/uPA receptor (uPAR) system is important in numerous physiological and pathological processes, particularly proliferation, adhesion and migration (Estreicher *et al*, 1990; Wei *et al*, 1994, 1996; Blasi and Carmeliet, 2002). The uPAR is a GPI-anchored protein that lacks a transmembrane domain. Thus, how the receptor performs these activities is a particularly compelling question. The biological functions of uPAR mainly rely on its interactions with other cell surface and transmembrane proteins. uPAR interacts with integrins (Wei *et al*, 1996; Yebra *et al*, 1996; Degryse *et al*, 2001), the low-density lipoprotein (LDL)-receptor-related protein (LRP) (Nykjaer *et al*, 1992; Czekay *et al*, 2001), the G-protein-coupled receptor FPRL1 (formyl peptide receptor, also known as lipoxin A4 receptor, LXA4R) (Resnati *et al*,

2002), the endothelium-derived growth factor receptor (Liu *et al*, 2002). Thus, the cellular response to uPA–uPAR binding depends on cell type, the nature of the mediating partner, the level of its expression, and the membrane environment. However, little is known about how the specificity of these interactions is attained and regulated.

Vascular smooth muscle cells (VSMC) migrate from the media into the intima, proliferate, synthesize extracellular matrix and form the neointima in a process called vascular ‘remodeling’. The uPA/uPAR signaling system participates in these processes (Clowes *et al*, 1990; Carmeliet *et al*, 1997; Okada *et al*, 1998). Others and we have shown that uPA increases VSMC migration (Stepanova *et al*, 1997) and proliferation (Dumler *et al*, 1999b). VSMC migration involves activation of the Janus kinase Tyk2, phosphatidylinositol-3-kinase (PI3-K) (Kusch *et al*, 2000) and the RhoA/Rho kinase pathway (Kiian *et al*, 2003). Activation of the transcription factor STAT1 is involved in uPA-associated changes of VSMC proliferation (Kunigal *et al*, 2003). However, the coupling mechanism between these signaling mediators and uPAR is unknown.

Platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR) is involved in atherosclerosis-associated vessel wall remodeling by modulation of VSMC migration and proliferation (Koyama *et al*, 1992; Sano *et al*, 2001). The two PDGFR (α and β) are expressed in VSMC, although PDGFR- β expression is higher (Kitami *et al*, 1995). PDGFR- β is particularly implicated in vascular remodeling (Sano *et al*, 2001).

Inter-relationships between the fibrinolytic system and PDGF/PDGFR has been reported in several studies (Reuning *et al*, 1996; Stepanova *et al*, 1997; Lau, 1999; Tanaka *et al*, 2002). Similar to the uPAR-directed signaling pathways in VSMC, PI3-K mediates PDGF-induced chemotaxis in various cell types (Kundra *et al*, 1994; Carlin *et al*, 2003; Puglianiello *et al*, 2004). The similarity of signaling pathways and functional responses to uPA/uPAR and PDGF/PDGFR suggested common signaling machinery. In the present study, we provide evidence that PDGFR- β is required for uPA to induce behavioral changes in human primary VSMC.

Results

PDGFR- β is required for uPA-induced VSMC migration and proliferation

We first performed experiments on cell migration when both uPA and PDGF-BB were present in the cell stimulation medium. Both agents induced cell migration in Boyden chamber; however, uPA strongly inhibited the PDGF-induced migration. We observed similar results using uPA amino-terminal fragment (ATF) (Figure 1A), indicating that the effect was independent of uPA catalytic activity. Addition of uPA or ATF also inhibited PDGF-BB-induced proliferation of VSMC as shown in Figure 1B. Next, we downregulated the uPAR expression and subsequently the PDGFR- β expression using siRNA duplexes introduced in VSMC by nucleofection

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Figure 1 Addition of uPA or ATF inhibits PDGF-BB-induced VSMC migration and proliferation. (A) VSMC migration stimulated by 25 nM uPA, 25 nM ATF, 20 ng/ml PDGF-BB: uPA + PDGF-BB or ATF + PDGF-BB was assessed in a Boyden chamber (1 a.u. is 0.39 ODu). (B) VSMC proliferation stimulated as in (A) was assessed by BrdU uptake.

technology. Control cells were nucleofected with the same amount of nonspecific siRNA duplexes. The downregulation of expression of both receptors 24 h after cell nucleofection was verified by Western blotting (Figure 2A and B). When the expression of uPAR was downregulated, addition of uPA did not stimulate VSMC migration and proliferation and did not lead to inhibition of PDGF-induced migration (Figure 2C and D). PDGF-BB-induced responses in those cells remained unchanged, which is consistent with earlier data (Herbert *et al.*, 1997). Similar results were obtained when cells were preincubated with R3 anti-uPAR antibodies. These antibodies are known to prevent uPA binding to uPAR (Ronne *et al.*, 1991) (Supplementary Figure 1A and B). VSMC with a reduced level of PDGFR- β demonstrated reduced migration and proliferation in the presence of PDGF-BB. Interestingly, stimulation of cell migration and proliferation by uPA was also significantly impaired. (Figure 2C and D). These findings suggest that PDGFR- β is required for uPA-induced signaling. The relatively high PDGF-induced response of cells lacking PDGFR- β is most likely explained by PDGF-BB binding to PDGFR- α . We observed that PDGF-AA induced migration and proliferation of VSMC, which is similar to observations made by others (Raines *et al.*, 1989; Ferns *et al.*, 1990) (Supplementary Figure 1C and D). When PDGFR- β expression was downregulated, PDGF-AA was still able to stimulate VSMC migration and proliferation. This fact indicates that PDGFR- β expression downregulation does not interfere with responses induced by PDGFR- α .

PDGFR- β tyrosine kinase activity is required for uPA-induced VSMC migration and proliferation; PDGFR- β is autophosphorylated in response to uPA

Our experiments with PDGFR silencing imply a PDGFR- β requirement for uPA-dependent VSMC behavior. Signaling events originating from PDGFR require its tyrosine kinase activity (Heldin and Westermark, 1999). We next tested whether or not the PDGFR tyrosine kinase is a decisive element in uPAR-induced signaling. We first used a pharmacological inhibitor of PDGFR kinase activity, tyrphostine AG1295 that has been shown to inhibit PDGFR kinase activity (Kovalenko *et al.*, 1994), and PDGF-induced proliferation and migration of VSMC (Banai *et al.*, 1998). First, we assayed PDGF-BB-induced VSMC migration and proliferation in the

Figure 2 uPAR is required for uPA-dependent inhibition of PDGF-BB-induced migration and proliferation; PDGFR- β is required for uPA-induced responses. Expression of uPAR (A) and PDGFR- β (B) 24 h after cell nucleofection with corresponding silencing RNA duplexes and cells nucleofected with corresponding amount of nonspecific RNA duplexes assessed by Western blotting. The lower panels blotted with anti-actin antibodies demonstrate equal loading of the gels. Migration (C) and proliferation (D) of VSMC nucleofected with noncoding, uPAR and PDGFR- β si-RNA duplexes are shown. Cells were stimulated with 25 nM uPA, 20 ng/ml PDGF-BB. Migration was measured in a wounding model. Proliferation was measured by BrdU uptake.

presence of AG1295 and checked for toxicity. AG1295 inhibited PDGF-BB-induced responses of VSMC and PDGFR- β phosphorylation in a dose-dependent manner (Supplementary Figure 2). VSMC treatment with 1–20 μ M AG1295 did not influence the number of viable cells, consistent with previously published data (Fishbein *et al.*, 2000). Further, we followed the uPA- and ATF-induced VSMC responses when cells were treated with the same concentrations of AG1295. The uPA- and ATF-induced VSMC migration and proliferation were dose-dependently inhibited by AG1295, whereas the basal levels of cell migration and proliferation were not affected (Figure 3A and B). AG1295 is known to also inhibit the c-Kit tyrosine kinase receptor (Kovalenko *et al.*, 1994). Since VSMC from the saphenous vein were reported to express c-Kit (Hollenbeck *et al.*, 2004), we tested whether or not VSMC from the coronary artery also express c-Kit. Similar to Hibbert *et al.* (2004), we did not observe any c-Kit expression in VSMC from the coronary artery. Therefore, the inhibitory effects of AG1295 can most likely be attributed to PDGFR inhibition.

We next expressed dominant-negative tyrosine kinase inactive mutant form of PDGFR- β (R634) in VSMC using cell nucleofection. The VSMC expressing the R634 mutant

Figure 3 PDGFR- β tyrosine kinase activity is required for uPA-induced responses. (A) VSMC migration was assessed in a Boyden chamber. Human VSMC were treated with indicated concentrations of AG1295 for 1 h and then allowed to migrate towards 25 nM uPA or 20 nM ATF (1 a.u. is 0.32 ODu). (B) VSMC proliferation was measured by BrdU uptake. Serum starved VSMC were stimulated as in (A) in the presence of indicated concentrations of AG1295. Migration (C) and proliferation (D) of VSMC expressing an R634 PDGFR- β mutant are shown. Migration was measured in a wounding model. Proliferation was measured by BrdU uptake. PDGFR- β was immunoprecipitated from VSMC stimulated with 10 ng/ml PDGF-BB (E) or 10 nM uPA (F), and the degree of its phosphorylation was assessed by Western blotting using anti-phosphotyrosine antibodies. The lower panels demonstrate equal amount of immunoprecipitated PDGFR- β .

showed impaired PDGFR phosphorylation in response to PDGF-BB (Supplementary Figure 3A and B). This finding indicates that PDGFR signaling was indeed reduced in those cells. Both migratory and proliferative responses to PDGF-BB and uPA were also impaired in R634-expressing cells (Figure 3C and D). These findings clearly demonstrate that the PDGFR tyrosine kinase activity is required to mediate uPA-induced responses in VSMC.

Since we found that uPA-induced signaling in VSMC depended on PDGFR kinase activity, we then investigated the PDGFR phosphorylation level in response to uPA stimulation. We immunoprecipitated PDGFR- β from lysates of nontreated and uPA-stimulated VSMC and assessed its phosphorylation

status by Western blotting. We detected increased PDGFR- β phosphorylation in uPA-treated cells, similar to cells treated with PDGF-BB (Figure 3E and F). The effect was observed at 5 min of uPA stimulation and decreased thereafter.

uPAR and PDGFR- β are associated in VSMC in an uPA-dependent manner

To obtain evidence for an association of uPAR and PDGFR- β , co-immunoprecipitation studies were performed. We immunoprecipitated PDGFR- β and detected uPAR in the immunoprecipitates with R3 anti-uPAR antibodies (Monozyme). We observed some association of the receptors in nonstimulated cells (Figure 4A). After 5 min of uPA stimulation, we found a mild increase in uPAR co-immunoprecipitation with PDGFR- β . Prolonged stimulation led to disassembly of receptors complexes. The identity of the observed band as uPAR was verified in additional experiments using polyclonal anti-uPAR antibodies (Santa Cruz) with or without the corresponding inhibitory peptide for Western blotting (Supplementary Figure 4A). To confirm the association between the receptors, we then performed immunoprecipitation with anti-uPAR antibodies. PDGFR- β was detected in immunoprecipitates using polyclonal antibodies (Upstate). We obtained similar results (Figure 4B) and found that uPAR was associated with PDGFR- β in an uPA-dependent manner. Identity of the observed band to PDGFR- β was also verified using other antibodies with or without the corresponding inhibitory peptide (Supplementary Figure 4B). However, when cells were stimulated with PDGF-BB, we observed no increased association of the receptors in response to stimulus, but only unchanged basal levels similar to those observed in cells before stimulation with uPA (Figure 4C).

uPAR-PDGFR- β complex formation was confirmed in chemical crosslinking experiments. uPA-stimulated cells were treated with the bifunctional chemical crosslinker disuccinimidyl suberate (DSS) followed by immunoprecipitation with anti-PDGFR- β antibody. The immunoprecipitated proteins were then separated by PAGE and detected with an anti-uPAR antibody. Two complexes, estimated at 240–260 and 380–400 kDa, were observed in the cells subjected to crosslinking (Figure 4D, left panel). Similar to the results of the co-immunoprecipitation above, the amount of crosslinked receptors increased after 5 min of uPA treatment. The presence of uPAR/PDGFR complexes in nonstimulated cells may be related to minimal basal autocrine VSMC stimulation by endogenously produced uPA (Clowes *et al*, 1990). From the molecular weights of the complexes, we assumed that the 240–260 kDa complex might represent monomeric PDGFR crosslinked to uPAR, and the 380–400 kDa complex might represent a PDGFR dimer crosslinked to uPAR. To confirm the composition of the complexes, the membranes were stripped and developed with anti-PDGFR antibodies. Similar bands were observed in this case (Figure 4D, right panel).

To provide further evidence for the association of uPAR and PDGFR- β , we used a recombinant PDGFR- β /Fc chimeric protein. This chimera contained the human PDGFR- β extracellular domain fused to the Fc fragment of human IgG. The chimera bound PDGF with high affinity and was a potent antagonist of PDGF-induced signaling and responses (Heldin and Claesson-Welsh, 1994). Since the chimeric protein had a different molecular weight than native PDGFR and was poorly recognized by the anti-PDGFR antibodies used for

Figure 4 uPAR and PDGFR- β in VSMC associate in an uPA-dependent manner. (A) PDGFR- β was immunoprecipitated from lysates of uPA-stimulated VSMC using rabbit polyclonal anti-PDGFR- β antibodies and uPAR in immunoprecipitates was visualized by Western blotting with R3 antibodies. (B) uPAR was immunoprecipitated from lysates of uPA-stimulated VSMC with R3 antibodies, and PDGFR- β in immunoprecipitates was visualized by Western blotting with rabbit polyclonal anti-PDGFR- β antibodies. (C) PDGFR- β was immunoprecipitated from lysates of PDGF-BB-stimulated VSMC, and uPAR in immunoprecipitates was visualized by Western blotting. (D) uPA-stimulated cells were subjected to crosslinking using 2.5 mM DSS, followed by immunoprecipitation with anti-PDGFR- β antibody. The immunoprecipitated proteins were then separated by PAGE and immunoblotted with anti-uPAR R3 antibody (left panel). Then the membranes were stripped and immunoblotted with anti-PDGFR- β antibodies (right panel). (E) VSMC were stimulated with uPA for 5 min in the presence of recombinant PDGFR/Fc chimera protein or nonspecific IgG, washed to remove unbound PDGFR/Fc, uPAR was immunoprecipitated and the amount of PDGFR- β in immunoprecipitates was analyzed by Western blotting.

Western blotting, the chimeric protein did not interfere with immunoprecipitation experiments. In our experiments, PDGF-induced phosphorylation of PDGFR in the presence of chimera was indeed significantly impaired. The impairment was shown by immunoprecipitation when recombinant PDGFR- β /Fc was added to the cell medium at a concentration

of 2 μ g/ml before a stimulus was applied. As expected, receptor phosphorylation was significantly impaired in the presence of the chimeric protein (Supplementary Figure 4C). We reasoned that PDGFR- β /Fc might be able to compete with endogenous PDGFR to form complexes with uPAR as well. Indeed, in the presence of PDGFR- β /Fc, we observed no uPA-induced increase of uPAR-PDGFR- β association (Figure 4E) in co-immunoprecipitation experiments.

Next, we used an immunofluorescence approach to confirm association of uPAR and PDGFR- β in VSMC. We stained nonstimulated and uPA-stimulated VSMC for uPAR and PDGFR- β and looked for their colocalization. The receptors did not colocalize in nonstimulated control cells (Figure 5). However, uPA stimulation for 5 min induced a transient increase in colocalization. These visual observations were confirmed by fluorograms. Distribution of the points in the fluorograms confirmed the absence of colocalization of the two receptors in nonstimulated control VSMC and cells stimulated with uPA for 35 min. In contrast, points of the fluorogram obtained from cells stimulated with uPA for 5 min were concentrated along the whole main diagonal of the quadrant, indicating colocalization of uPAR and PDGFR- β .

uPA stimulation leads to PDGF-independent PDGFR- β dimerization

Our results suggest involvement of catalytically active PDGFR in uPA-induced signaling. We speculated that uPA stimulation might cause release of PDGF and thereby activate PDGFR indirectly. VSMC express primarily PDGF-AA and also PDGF-BB. We therefore studied the release of both PDGF forms. VSMC were stimulated with uPA for 0–30 min, the medium was collected, concentrated 20-fold, and PDGF content was studied with ELISA and Western blotting. We observed no detectable amount of PDGF in concentrated stimulation medium in any case. Since the detection limit for ELISA is below 30 pg/ml and since we observed no PDGF in 20-fold concentrated medium, we concluded that PDGFR activation in response to uPA is probably achieved via a distinct mechanism.

In crosslinking experiments, we identified a complex similar in size with dimeric PDGFR crosslinked to uPAR. We therefore studied whether or not uPA leads to PDGFR dimer formation in the absence of PDGF. We studied dimer formation with an immunoprecipitation approach under the conditions described by Li and Schlessinger (1991). We observed PDGFR- β bands at about 360 kDa corresponding to the molecular weight of the receptor dimer (Figure 6A). Dimer formation was strongly increased by uPA treatment. PDGF-BB stimulation, used as a positive control, led to a similar increase in receptor dimerization. Tyrosine phosphorylation of the 360 kDa bands was also highly induced by PDGF-BB and uPA, confirming activation of PDGFR in response to uPA stimulation (Figure 6B).

PDGFR dimerization was also studied by Fluorescence Resonance Energy Transfer (FRET) method. Images of an unstimulated cell and a cell stimulated with uPA (or PDGF-BB as a positive control) are shown in Figure 6C, left panels. As described in Materials and methods, the normalized FRET signal (F_N) distributions over cells are shown by color coding in the middle panels. For each cell, the right panel shows a histogram of the F_N signal distribution over the cell pixels. Each of the histograms is normalized by the total count of the corresponding cell pixels. As can be seen in the color-coded

Figure 5 uPAR and PDGFR- β in VSMC colocalize in an uPA-dependent manner. VSMC nonstimulated and stimulated with 10 nM uPA for the indicated time periods were immunostained with anti-PDGFR monoclonal antibodies and Alexa 488-conjugated secondary antibodies and anti-uPAR polyclonal antibodies visualized by Alexa 633-conjugated secondary antibodies. The frame size of all the images is $130 \times 130 \mu\text{m}$. The fluorograms were obtained by plotting each pixel of the overlay Alexa 488/Alexa 633 color fluorescence cell image. Pixel values of Alexa 488 and Alexa 633 fluorescence images represent horizontal and vertical axis values for each point, respectively. The points of the fluorograms are colored in accordance with count of pixels with specific Alexa 488 and Alexa 633 fluorescence intensities. The pixel count is shown in logarithmic scale.

distributions, only a very weak FRET signal was observed for the unstimulated cell, while strong FRET signals were seen for the uPA- or PDGF-stimulated cells. Quantitative estimation of the FRET signal can be obtained from the histograms in the right panels. The peak of the histogram obtained from the unstimulated cell is indistinguishable from the zero indicating only a weak FRET signal. The histograms obtained from the uPA- and PDGF-stimulated cells show peaks well separated from the zero, with normalized FRET peak values of about 0.18 and 0.27, respectively. From these experiments, we can conclude that uPA stimulation leads to PDGF-independent dimerization of PDGFR- β in VSMC.

Tyrosine kinase activity of PDGFR- β and its association with uPAR are required for uPA-induced RhoA activation, and STAT1 activation and nuclear translocation

Earlier, we found that the PI3-K/RhoA pathway mediates the uPA-directed migratory capacity of VSMC (Kusch *et al*, 2000; Kiyan *et al*, 2003). We next studied whether or not RhoA

activation in response to uPA also required PDGFR- β . First, we measured RhoA activation upon inhibition of PDGFR tyrosine kinase activity by AG1295, using a pulldown assay. We treated VSMC with $10 \mu\text{M}$ AG1295. This treatment prevented uPA-induced RhoA activation (Figure 7A). We then overexpressed mutant forms of PDGFR. RhoA activation in response to uPA was impaired in VSMC expressing kinase inactive R634 mutant (Figure 7B). Similar results (Figure 7B) were obtained with cells expressing an F740/F751 PDGFR mutant form that lacks tyrosine residues required for PI3-K binding (Tyr740/Tyr751 changed to Phe). PDGF-induced Akt activation was impaired in those cells, verifying a lack of PDGFR association with PI3-K (Supplementary Figure 5). Taken together, these data suggest that PDGFR- β and its association with PI3-K are required for uPA-induced RhoA activation.

Next, we studied whether or not association of uPAR and PDGFR- β is required for RhoA activation. As we observed earlier, a PDGFR/Fc chimera interfered with formation of the uPAR-PDGFR- β complex in immunoprecipitation experi-

Figure 6 uPA induces dimerization of PDGFR. VSMC were stimulated with uPA or PDGF-BB, then PDGFR was immunoprecipitated and the degree of receptor dimerization (A) and dimer phosphorylation (B) was assessed by Western blotting. (C) Serum-starved VSMC were stimulated for 5 min with uPA or PDGF-BB, then washed with cold PBS, fixed with 1% paraformaldehyde in PBS for 10 min at 4°C and labeled with mixture of Alexa 488- and Alexa 594-conjugated anti PDGFR- β antibodies, 5 μ g/ml of each conjugate in PBS/1% BSA overnight at 4°C. The left panels show images of cells stained for PDGFR- β . The middle panels show the normalized FRET signal (F_N) distributions over cells indicated by color coding. The right panels show histograms of the F_N signal at the cell pixels.

Figure 7 PDGFR- β and its association with uPAR and PI3-K are required for uPA-induced RhoA activation and VSMC migration. (A) RhoA activation in response to uPA was measured by pull-down assay after VSMC pretreatment with 10 μ M AG1295 or a corresponding amount of DMSO. (B) uPA-induced RhoA activation was measured in VSMC expressing either R634 PDGFR- β or F740/F751 PDGFR- β . (C) RhoA activation was measured in VSMC preincubated with 2 μ g/ml PDGFR- β /Fc chimera protein prior to uPA stimulation. Migration (D) and proliferation (E) of VSMC expressing F740/F751 PDGFR- β mutant. Migration was measured in a wounding model. Proliferation was measured by BrdU uptake. Cells were stimulated by 25 nM uPA, 20 ng/ml PDGF-BB.

ments. In the presence of chimeric protein, RhoA activation was also significantly impaired (Figure 7C).

In agreement with our earlier data (Kusch *et al*, 2000; Kiyan *et al*, 2003), uPA-induced VSMC migration was completely abolished in cells overexpressing the F740/751 PDGFR mutant (Figure 7D). PDGF-induced migration was also impaired in these cells, confirming a requirement of PDGFR association with PI3-K for PDGF-induced VSMC migration. In contrast, the uPA-induced cell proliferation was less affected (Figure 7E). These findings clearly show that PDGFR tyrosine kinase activity is necessary for the induction of the uPA-induced signaling cascade towards RhoA activation and cell migration.

VSMC stimulation with uPA is accompanied by transient activation and nuclear translocation of STAT1 (Dumler *et al*,

Figure 8 uPA-dependent STAT1 nuclear translocation requires active PDGFR- β . VSMC were pretreated with 10 μ M AG1295 for 1 h, then stimulated with 10 nM uPA for 35 min and immunostained with anti-STAT1 polyclonal antibodies visualized by Alexa 488-conjugated secondary antibodies. The frame size of all the images is 240 $\times$ 240 μ m.

1998, 1999a). To investigate the role of PDGFR- β in STAT1 activation, we first tested whether or not PDGFR- β tyrosine kinase activity was required for STAT1 nuclear translocation. We used an immunocytochemical approach to follow STAT1 nuclear translocation in response to uPA. To inhibit PDGFR kinase activity, VSMC were treated with AG1295 for 1 h, stimulated with uPA and stained with anti-STAT1 antibodies and Alexa 488-labeled secondary antibodies. The uPA stimulation led to significant nuclear translocation of STAT1 consistent with our previous observations (Dumler *et al*, 1998, 1999a). However, when PDGFR tyrosine kinase was inhibited, there were no significant changes in STAT1 intracellular localization in response to uPA (Figure 8).

STAT1 dimerization and nuclear translocation requires phosphorylation on tyrosine residues. We next asked whether or not PDGFR- β is required for the uPA-induced Tyr701 phosphorylation of STAT1. STAT1 Tyr701 phosphorylation was impaired by both pharmacological inhibition and the R634 kinase inactive PDGFR mutant expression (Figure 9A and B). R634-expressing VSMC showed slight increase in the basal level of STAT1 phosphorylation. However, the uPA-induced increase in STAT1 phosphorylation was abolished. The STAT1 hyperphosphorylation in R634-expressing cells might be a result of a dysfunction in STAT1 dephosphorylation by the phosphatase SHP-2 in a PDGFR-dependent manner (our unpublished data). In contrast, we found that STAT1 phosphorylation was normal in cells overexpressing F740/751 PDGFR- β mutant (Figure 9C). This finding suggests that the PI3-K/RhoA pathway is probably not involved in STAT1 phosphorylation. Interfering with uPAR-PDGFR- β complex formation using the PDGFR- β /Fc chimera protein had a similar effect as did PDGFR inhibition and prevented STAT1 phosphorylation (Figure 9D).

Discussion

Among the processes occurring in the arterial wall during atherosclerosis and postangioplasty restenosis, a pivotal role

Figure 9 uPA-induced STAT1 (Tyr701) phosphorylation requires active PDGFR- β . (A) Nontreated VSMC and cells pretreated with 10 μ M AG1295 were stimulated with 10 nM uPA for indicated time. STAT1 (Tyr701) phosphorylation was assessed by Western blotting with anti-phospho-(Tyr701) STAT1 antibodies. The lower panels demonstrate equal protein loading of the gels. uPA-dependent STAT1 (Tyr701) phosphorylation in VSMC expressing R634 (B) and F740/751 (C) mutants, measured by Western blotting with anti-phospho-(Tyr701) STAT1 antibodies. The lower panels demonstrate equal protein loading of the gels. (D) STAT1 phosphorylation in response to uPA measured in VSMC preincubated with 2 μ g/ml PDGFR- β /Fc chimera protein prior to uPA stimulation. The lower panel demonstrates equal protein loading of the gel.

is played by VSMC migration and proliferation. These processes are regulated by many complex signaling pathways within VSMC. One of these pathways involves regulation of the fibrinolytic uPA/uPAR system orchestrating several different, and in some cases interfering, cellular functions, such as regulation of cell-associated proteolysis, cell adhesion, migration, proliferation, and differentiation (Blasi and

Carmeliet, 2002). As uPAR is linked to the outer membrane leaflet by a GPI-anchor and is devoid of any known catalytic activity, all of its diverse biological functions depend strictly on its cell-specific interactions with other proteins. PDGFR- β is involved in vascular remodeling (Sano *et al*, 2001) and wound healing (Heldin and Westermark, 1999). The reported interplay between the PDGF/PDGFR- β and the uPA/uPAR systems, and the similarities in their signaling machineries, prompted us to hypothesize that PDGFR- β may serve as a transmembrane adaptor for uPAR in VSMC. We showed that in human VSMC, uPA binding by uPAR induces its association with PDGFR- β , which results in PDGFR- β activation and receptor dimerization. We documented association of uPAR and PDGFR in co-immunoprecipitation studies, chemical crosslinking experiments, and by immunocytochemistry. PDGFR dimerization was demonstrated by immunoprecipitation and FRET analysis. Since we did not observe any release of PDGF after uPA stimulation, we assume that PDGFR dimerization occurs independently of the presence of its ligand. The tyrosine kinase activity of PDGFR- β is decisive for mediating downstream signaling. Interfering with complex formation of the receptors also prevents uPA-induced signaling in VSMC. Similar mechanisms are activated in VSMC by ATF of uPA and therefore are independent of the catalytic activity of uPA.

The Jak/STAT pathway is the main signaling pathway induced by uPA in VSMC. The migratory response is mediated by the Tyk2/PI3-K/RhoA/Rho kinase pathway. We found that the latter pathway requires association of uPAR with PDGFR- β and depends on its kinase activity and its interactions with PI3-K via Tyr740/Tyr751. Transient STAT1 activation by uPA also requires association of the receptors and PDGFR kinase activity, but is independent of PI3-K. PDGFR- β is capable of interacting with STAT1 and phosphorylates STAT1 directly (Vignais and Gilman, 1999). The same mechanism might be utilized in VSMC. As previously reported (Bromberg *et al*, 1998), transient activation of STAT1 might be associated with regulation of cell proliferation. Alternatively, the main role of STAT1 activation may be to increase the time for VSMC migration towards uPA by transiently slowing down cell cycle progression (Dimberg *et al*, 2003).

Studying VSMC migration, we observed uPA-dependent inhibition of PDGF-BB-induced migration. One possible explanation for this observation is that uPAR competes for PDGFR- β in order to induce uPA-stimulated signaling. The requirement of uPAR for uPA-dependent inhibition of PDGF-BB-induced migration observed in our uPAR downregulation experiments favors this assumption. However, other explanations are also possible. Thus, increased internalization and catabolism of receptors complexes via LRP1B may take place in the presence of both ligands (Tanaga *et al*, 2004). Alternatively, autocrine upregulation of PDGF-AA expression by VSMC may inhibit cell migration towards PDGF-AB-, -BB, and other chemoattractants (Koyama *et al*, 1992). Our data conflict with the finding that uPA and PDGF have an additive effect on airway SMC migration (Carlin *et al*, 2003). However, in that study uPA alone did not lead to increased migration. These discrepancies confirm the known cell specificity in responses to uPA. They point to significant biological differences in airway smooth muscle cells and VSMC from coronary artery that may explain different behavioral responses.

Our findings elucidate a functional link between uPAR and signaling cascades induced in VSMC by uPA in order to regulate cell migration and proliferation. These findings provide a molecular basis for mechanisms utilized by human VSMC to induce uPAR- and PDGFR-directed signaling and regulated cell functions in vascular remodeling.

Materials and methods

Reagents and antibodies

High-quality commercial grade chemicals were purchased from Sigma, Amersham Pharmacia Biotech, and Merck. Chemiluminescent signal enhancers were obtained from NENTM Life Science Products, Inc. AG1295 was purchased from Calbiochem. DSS was purchased from Sigma. uPA was purchased from Loxo, urokinase ATF was from Innovative research. PDGFR-Fc chimera protein was from R&D Systems. Grid coverslips (175 μ m grid size) were from Eppendorf. Monoclonal anti-PDGFR- β antibodies were from R&D Systems; polyclonal anti-PDGFR- β antibodies and monoclonal anti-phosphotyrosine antibodies were from Upstate; monoclonal anti-uPAR (clone R3) antibodies were from Monozyme. Monoclonal anti-RhoA antibodies, polyclonal anti-uPAR, and anti-PDGFR- β antibodies with corresponding inhibitory peptides were from Santa Cruz Biotechnology, Inc.; monoclonal anti-STAT1 antibodies were from Transduction Laboratories; polyclonal anti-phospho-(Tyr⁷⁰¹)-STAT1 antibodies, polyclonal anti-phospho-Akt, and polyclonal anti-Akt antibodies were from Cell Signaling; fluorescent Alexa 488- and Alexa 633-conjugated secondary antibodies were from Molecular probes, Inc.; and peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc.

Cell culture, plasmid construction, and transfection

Primary human coronary artery VSMC were obtained from Cambrex, Inc. and from Promocell GmbH; the cells were grown in a medium recommended by the suppliers and were used between passages 3 and 7.

Dr Andrius Kazlauskas (Schepens Eye Research Institute, Harvard Medical School, Boston, MA) has kindly given us vectors for overexpression of PDGFR- β mutant forms: tyrosine kinase inactive R634 (Lys 634-Arg); defective in PI3-K binding F740/F751 (Tyr 740, Tyr751-Phe). *EcoRI/BamHI* fragments containing mutated PDGFR- β sequence were subcloned into *EcoRI/BamHI*-digested pMACS vector (Miltenyl Biotec). Dr MA Schwartz (Department of Vascular Biology, The Scripps Research Institute, La Jolla, CA) kindly gave us the plasmid expressing the GST fusion protein with rhotekin Rho binding domain (GST-TRBD). RNAsi duplexes to downregulate expression of uPAR and PDGFR- β , as well as control duplexes were purchased from Santa Cruz Biotechnology, Inc.

Human primary VSMC were transfected using NucleofectorTM (Amaya Biosystems, Inc.). Basic Primary Smooth Muscle cell nucleofector kit (Amaya Biosystems, Inc.) was used according to the manufacturer's instructions. Time curve for overexpression of every protein was performed using Western blotting and it was typically between 24 and 48 h after nucleofection. In case of siRNA duplexes, nucleofection was performed as recommended for aortic smooth muscle cells except that RNA (3 μ g per nucleofection sample 10^6 cells) was used.

Immunoprecipitation, RhoA pulldown assay, and Western blotting

Subconfluent and serum-starved VSMC were treated with 10 nM uPA for 1–15 min at 37°C and lysed with buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM Na₃VO₄, 1 mM NaF directly on culture dishes. Lysates were clarified by centrifugation at 10 000 r.p.m. for 10 min and were used for immunoprecipitation. Typically, 5 μ g of antibodies were incubated with 600–1000 μ g of cell lysate protein for 2 h, then protein A/G agarose was added and incubation proceeded for another hour. Then beads were washed 3 times with 500 μ l PBS containing 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM Na₃VO₄, 1 mM NaF, solubilized in Laemmli SDS sample buffer and then analyzed by Western blotting.

When immunoprecipitation was performed in the presence of PDGFR-Fc chimera protein, it was added to cell stimulation medium at concentration 2 μ g/ml. After cells were stimulated with either PDGF-BB or uPA, unbound chimera protein was removed by extensive cell monolayer washing with ice-cold tris-buffered saline solution, cells were lysed, and PDGFR was immunoprecipitated. Level of PDGFR phosphorylation was assessed by Western blotting with anti-phosphotyrosine antibody.

For RhoA pulldown assay, lysates of uPA-stimulated VSMC were incubated with agarose-conjugated GST-TRBD for 45 min at 4°C with constant agitation as described previously (Kiian *et al*, 2003). After incubation, beads were washed, solubilized in Laemmli SDS sample buffer, and analyzed by Western blotting.

Chemiluminescent images were captured using VersaDoc-3000 (Biorad Laboratories) and quantified using Quantity One software (Biorad Laboratories).

Crosslinking

For crosslinking, cells were stimulated with 10 nM uPA, washed and lysed as described above (instead of Tris, 20 mM HEPES was used for a lysis buffer), and crosslinked by the addition of 2.5 mM DSS for 30 min at room temperature. The crosslinking reaction was stopped by adding methylammonium chloride to a final concentration of 70 mM and the samples were used for immunoprecipitation and Western blotting.

Chemotaxis assay, wound healing assay, and proliferation assay

Chemotaxis assay was performed using modified Boyden chambers with collagen I-coated polyvinylpyrrolidone-free polycarbonate filter membranes, 8 μ m pore size as described previously (Kusch *et al*, 2000). uPA and PDGF-BB were added in concentrations 25 nM and 20 ng/ml, respectively, to the lower wells of the Boyden chamber. Cell migration was quantified by densitometry of the stained membrane, using VersaDoc-3000 (Biorad Laboratories) and Quantity One software (Biorad Laboratories). Migration of untreated unstimulated cells was taken as 1 a.u. To assess VSMC migration in wound healing model using grid coverslips, a confluent serum-starved VSMC monolayer was wounded as described previously (Kusch *et al*, 2000) and medium containing either 25 nM uPA or 20 ng/ml PDGF-BB was added to the cells. At 24–36 h after wounding, the migrated cells in the grid area were counted. Average migrated cell number $\pm$ standard error (s.e.) was calculated from three independent experiments.

VSMC proliferation was quantified using Cell Proliferation ELISA kit (colorimetric) from Roche Applied Science, based on the measurement of BrdU incorporation during DNA synthesis in accordance to the manufacturer's instructions. uPA and PDGF-BB were added in concentrations 25 nM and 20 ng/ml, respectively. Average OD at 450 nm $\pm$ s.e. calculated from three independent experiments is indicated.

Immunofluorescence confocal microscopy

Cells were seeded and cultured on glass coverslips. Serum-starved VSMC were treated for an appropriate time period with 10 nM uPA. For STAT1 immunocytochemical staining, cells were pretreated either with 10 μ M AG1295 or a corresponding amount of DMSO prior to uPA stimulation. After incubation with uPA, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. For STAT1 immunocytochemical staining, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. For PDGFR/uPAR staining, cells were used without permeabilization. After overnight incubation at 4°C with 1% bovine serum albumin in PBS, the preparations were washed 3 times with PBS. Incubations with each primary antibody were performed for 2 h at room temperature in 1% bovine serum albumin in PBS. Incubations with all fluorescent-labeled secondary antibodies were performed for 1 h. After staining, cells were embedded in Poly-Mount mounting media (Polysciences, Inc.). The fluorescence cell images were captured using a Leica TCS-SP2 AOBs confocal microscope (Leica Microsystems). All the images were taken with oil-immersed $\times$ 63 objective, NA = 1.4.

FRET analysis

Monoclonal anti-PDGFR- β antibodies were directly conjugated to Alexa 488 and Alexa 594 using antibody labeling kits from Molecular Probes, Inc. as recommended by the manufacturer. The

final coupling ratio was determined accordingly to the instructions and was 3.2 and 3.3 for Alexa 488- and Alexa-594-conjugated antibodies, respectively. Isotypic IgG labeled with the same procedure gave no detectable immunostaining. Images were acquired using a Leica TCS SP2 AOBS Confocal Microscope with HCX PL APO CS 63.0 $\times$ 1.40 oil objective. Images were recorded in the donor and acceptor channels with detection wavelengths range set to 505–575 and 610–750 nm, respectively. All the images were acquired by scanning the area of 130 $\times$ 130 μm^2 with resolution of 1024 $\times$ 1024 pixels. Prior to any data processing, the background noise level was subtracted from the pixel values for all the images. The background noise levels were obtained for the donor and acceptor channels by acquiring and averaging of 100 images in each of the channels with the excitation lasers off and microscope settings the same as for image acquisition.

The images of cells were recorded in the donor and acceptor channels under the excitation of 488 nm radiation from Ar-laser. The obtained pixel values after the subtraction of noise level are denoted as D_{488} and A_{488} , respectively. Then the image of the same cell was recorded in the acceptor channel under the excitation by 594 nm radiation from HeNe-laser. The obtained pixel values after the subtraction of noise level is denoted as A_{594} .

Following Chamberlain *et al* (2000), the A_{488} signal is a sum of net FRET signal and fluorescence signals produced by each of the individual fluorophores in the acceptor channel. The latter contributions from the Alexa 488 and Alexa 594 fluorophores were calculated and subtracted from A_{488} signal in order to obtain net FRET signal $F = A_{488} - \delta D_{488} - \epsilon A_{594}$, where δ is the bleedthrough factor for Alexa 488, and ϵ is the bleedthrough factor for Alexa 594.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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