Soluble M6P/IGF2R Released by TACE Controls Angiogenesis via Blocking Plasminogen Activation

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Rationale: The urokinase plasminogen activator (uPA) system is among the most crucial pericellular proteolytic systems associated with the processes of angiogenesis. We previously identified an important regulator of the uPA system in the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R).

Objective: Here, we wanted to clarify whether and how did the soluble form of M6P/IGF2R (sM6P/IGF2R) contribute to modulation of the uPA system.

Methods and Results: By using specific inhibitors and RNA interference, we show that the tumor necrosis factor α convertase (TACE, ADAM-17) mediates the release of the ectodomain of M6P/IGF2R from human endothelial cells. We demonstrate further that sM6P/IGF2R binds plasminogen (Plg) and thereby prevents Plg from binding to the cell surface and uPA, ultimately inhibiting in this manner of Plg activation. Furthermore, peptide 18-36 derived from the Plg-binding site of M6P/IGF2R mimics sM6P/IGF2R in the inhibition of Plg activation and blocks cancer cell invasion in vitro, endothelial cell invasion in vivo, and tumor growth in vivo.

Conclusions: The interaction of sM6P/IGF2R with Plg may be an important regulatory mechanism to inhibit migration of cells using the uPA/uPAR system. (Circ Res. 2011;108:00-00.)

Key Words: fibrinolysis ■ cell migration ■ angiogenesis ■ tumor invasion

During tumor-associated angiogenesis, pericellular proteolytic systems are indispensable for endothelial cells to degrade surrounding matrix and sprout into new blood vessels. Similar machineries are hired by metastatic cells to invade tissues. Therefore, understanding of how these forces are regulated is crucial in recognizing new targets and establishing novel strategies for antiangiogenesis and anticancer therapy.1

The urokinase plasminogen activator (uPA) system is well known to mediate cell surface proteolysis in both endothelial and cancer cells.2 After binding to the uPA receptor (uPAR, CD87), inactive single-chain pro-urokinase is processed to double-chain urokinase (uPA), the central plasminogen (Plg) activator on the cell surface. The active serine protease plasmin facilitates breakdown of extracellular matrix components both directly and through the activation of matrix metalloproteinases (MMP).3 Vascular endothelial growth factor (VEGF), a pivotal proangiogenic factor induces activation of pro-urokinase and redistribution of uPAR to the leading edge of migrating endothelial cells.4 Moreover, uPAR is located in the leading edge of invading cancer cells.5 Thus, the uPA system plays a key role in tumor progression and specific targeting of this system has been recognized as a promising approach to treat cancer diseases.6 We have previously shown that the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R, CD222) inhibits uPAR functions in cell surface proteolysis and cell migration.7 Furthermore, we have proposed a mechanism whereby membrane-associated M6P/IGF2R controls cell invasion by regulating αv integrin expression and by accelerating uPAR cleavage leading to loss of the uPA-binding site on uPAR.8 Here, we put forward one more pathway through which M6P/IGF2R contributes to inhibition of the uPA system. The tumor necrosis factor α convertase (TACE, ADAM17) mediates the release of the ectodomain of M6P/IGF2R. This soluble form (sM6P/IGF2R) binds to Plg and, through blocking the interaction between Plg and uPA, ultimately inhibits pericellular Plg activation. We show further that a peptide derived from the Plg-binding site of M6P/IGF2R mimics sM6P/IGF2R in the inhibition of Plg activation and blocks cancer cell invasion in vitro, endothelial cells invasion in vivo and tumor growth in vivo.
The Release of sM6P/IGF2R Is Mediated by TACE

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

RNA Interference
To silence the expression of TACE, we transiently transfected primary human umbilical vein endothelial cells (HUVECs) with small interfering (si)RNA according to the instructions of the manufacturer.

Immunobiological Techniques
SDS-PAGE, immunoblotting, blue native electrophoresis (BN-PAGE), protein purification, gel filtration, in vitro binding, immunoprecipitation, and plasmin activity assays were performed as described previously with some modifications.

In Vitro and In Vivo Assays
Cell invasion, in vitro endothelial cell tube formation, in vivo angiogenesis Matrigel plug assays, and the chimeric human/mouse angiogenesis mouse embryonic fibroblasts for the presence of sM6P/IGF2R. We tested also the supernatants of wild type and TACE−/− mouse embryonic fibroblasts for the presence of sM6P/IGF2R. In wild type cells, the shedding was again reduced by GM6001. As expected, in TACE−/− cells, the release of sM6P/IGF2R was significantly, but not completely, reduced (≈80% reduction) (Figure 1D and 1E). These results provide strong evidence that TACE is involved in the proteolytic release of the M6P/IGF2R ectodomain; however, a possible contribution from other mechanisms cannot be excluded.

Interactions of sM6P/IGF2R With Plg
Although there are several studies demonstrating soluble forms of M6P/IGF2R in the circulation of rat and human, the function of sM6P/IGF2R has not been resolved. We characterized previously the membrane form of cellular M6P/IGF2R as a receptor for Plg. Because Plg is an abundant serum protein, we asked whether sM6P/IGF2R might bind serum Plg too and affect its function. To explore this possible functional association, we attempted to coimmunoprecipitate these 2 proteins from human serum. It was reported that the level of sM6P/IGF2R in rat serum increased on liver damage. Similarly, we found increased levels of sM6P/IGF2R in sera from patients with various liver disorders (Online Figure II), and we used these serum samples for our study.

We coprecipitated serum Plg with the anti-M6P/IGF2R monoclonal antibody (mAb) MEM-240; and vice versa, we coprecipitated sM6P/IGF2R with the anti-Plg mAb 4Pg (Figure 2A). MEM-240 recognizes an epitope within the extracellular repeat domains 6 to 9 of M6P/IGF2R; 4Pg recognizes an epitope within the amino-terminal region of M6P/IGF2R.11,12 Shorter fragments were not detected. We confirmed the specificity of the band by checking the supernatants derived from immortalized human endothelial cells (HUVECTert8) with normal and silenced expression of TACE. Transfection of HUVECs with one of the 2 TACE-specific siRNAs (TACEγ) resulted in a significant reduction of TACE expression and this downregulation correlated significantly with the reduced release of sM6P/IGF2R (Figure 1C and 1E). We did not detect any expression of MMP-8 at the protein level by immunoblotting analysis (data not shown). Regardless, we performed the silencing procedure of MMP-8 that, expectedly, had no effect on shedding. Notably, an unspecific inhibition of the TACE-mediated shedding by MMP-8 inhibitor I was already reported also by others.11 As a further proof of the involvement of TACE in the shedding of sM6P/IGF2R, we tested also the supernatants of wild type and TACE−/− mice embryonic fibroblasts for the presence of sM6P/IGF2R. In wild type cells, the shedding was again reduced by GM6001. As expected, in TACE−/− cells, the release of sM6P/IGF2R was significantly, but not completely, reduced (≈80% reduction) (Figure 1D and 1E). These results provide strong evidence that TACE is involved in the proteolytic release of the M6P/IGF2R ectodomain; however, a possible contribution from other mechanisms cannot be excluded.

We tested various protease inhibitors for their ability to block the release of sM6P/IGF2R and found that the broad MMP inhibitor GM6001 (galardin), the TACE inhibitor TAPI and the MMP-8 inhibitor I blocked the shedding completely (Figure 1B and 1E). Shedding was partially blocked by the MMP-3 inhibitor II; however, this was attributable to its toxic effect on HUVECs. Because the inhibitors are relatively nonspecific, we expressed in HUVEC siRNA constructs targeting 2 messenger RNA regions of TACE. Transfection of HUVECs with one of the 2 TACE-specific siRNAs (TACEγ) resulted in a significant reduction of TACE expression and this downregulation correlated significantly with the reduced release of sM6P/IGF2R (Figure 1C and 1E). We did not detect any expression of MMP-8 at the protein level by immunoblotting analysis (data not shown). Regardless, we performed the silencing procedure of MMP-8 that, expectedly, had no effect on shedding. Notably, an unspecific inhibition of the TACE-mediated shedding by MMP-8 inhibitor I was already reported also by others.11 As a further proof of the involvement of TACE in the shedding of sM6P/IGF2R, we tested also the supernatants of wild type and TACE−/− mice embryonic fibroblasts for the presence of sM6P/IGF2R. In wild type cells, the shedding was again reduced by GM6001. As expected, in TACE−/− cells, the release of sM6P/IGF2R was significantly, but not completely, reduced (≈80% reduction) (Figure 1D and 1E). These results provide strong evidence that TACE is involved in the proteolytic release of the M6P/IGF2R ectodomain; however, a possible contribution from other mechanisms cannot be excluded.

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We scrutinized the Plg-sM6P/IGF2R complex in serum by BN-PAGE, which allowed a complex dissection by the
Figure 1. Shedding of M6P/IGF2R is mediated by TACE. A, HUVECs were cultivated as described in Methods. After the indicated time intervals, samples were taken from supernatants for immunoblotting analysis (IB) by using a 7% SDS-PAGE gel, the anti-M6P/IGF2R mAb MEM-238, and chemiluminescence for development of the blot. B, HUVECs were cultivated 24 hours in the presence of various inhibitors: aprotinin (Apr) (100 μg/mL), leupeptin (Leu) (10 μg/mL), GM6001 (GM) (10 μmol/L), MMP-2/MMP-9 inhibitor I (M29i) (10 μmol/L), GM6001 (GM) (10 μmol/L), GM6001 (GM) (10 μmol/L), MMP-3 inhibitor II (M3i) (10 μmol/L), TAPI (10 μmol/L), TMPI (10 μmol/L), and chemiluminescence for development of the blot. The control level (medium without inhibitors) was set to 100% in each experiment. *P<0.005, **P<0.001 as indicated (n=3 experiments). C, Subconfluent HUVECs were transfected in a 48-well plate with the indicated siRNA constructs targeting human TACE (TACE1 and TACE2), MMP-8, and control siRNA (ctr) as described in Methods. Assays were performed 48 hours after transfection when medium was exchanged, and after an additional 24 hours, supernatant samples were taken for analysis as in A. D, Supernatants of wild-type and TACEKO (TACEKO) mouse embryonic fibroblasts cultivated 24 hours in the absence or presence of GM6001 (GM) (10 μmol/L) were analyzed by using the goat polyclonal anti-M6P/IGF2R Ab. As a control of similar cell densities, cell lysates were analyzed for cellular M6P/IGF2R, TACE, and uPAR. E, Statistical evaluation of the data presented in B through D. After analysis of respective supernatant samples, the intensities of the bands corresponding to sM6P/IGF2R were assessed by the chemiluminescence image analyzer FUJIFILM/LAS-4000 and the band density analysis software MultiGauge V3.0. At least 3 experiments were involved in calculations. The control level (medium without inhibitors) was set to 100% in each experiment. *P<0.05, **P<0.005 as indicated (n=3).
form of M6P/IGF2R, binds Plg through the amino-terminal part of domain 1. Finally, various shorter soluble forms occur in serum and conditioned media probably representing proteolytic fragments of sM6P/IGF2R.10,28 We tested whether plasmin per se could be responsible for the fragmentation of sM6P/IGF2R. Indeed, when we subjected purified M6P/IGF2R to in vitro proteolysis, plasmin but not uPA enabled its proteolytic processing (Online Figure III). These data indicate that a proteolytic processing of M6P/IGF2R may yield shorter soluble fragments. Notably, we also precipitated a shorter form (~50 kDa) from serum (Figure 2A). It is feasible that the shorter sM6P/IGF2R fragments which still convey the Plg-binding site might be able to bind Plg and be of functional relevance.

sM6P/IGF2R and Peptide 18-36 Have a Similar Negative Regulatory Effect on Plg Activation

Plg binding to the cell surface is a prerequisite for its controlled activation.29 Based on this assumption and the data presented above, it was conceivable that sM6P/IGF2R could be affecting Plg activation; and that peptide 18-36, derived from the Plg-binding region of M6P/IGF2R, could be a suitable modulatory tool to test this hypothesis. Therefore, we

Figure 2. sM6P/IGF2R complexes Plg in human serum. A, Human serum sample was diluted 20 times with PBS and subjected to immunoprecipitation (IP) using either mAbs against Plg (4Pg, 7Pg) or M6P/IGF2R (MEM-238, MEM-240) or an irrelevant control mAb (ctr). Precipitates were analyzed by immunoblotting (IB) using anti-Plg mAb 7Pg and goat polyclonal anti-M6P/IGF2R Ab and chemiluminescence for development. B, Human serum sample was diluted 10 times with PBS and analyzed by BN-PAGE in the first dimension followed by SDS-PAGE in the second dimension as described in Methods. Coomassie brilliant blue (CBB) stained ferritin (monomer 440 kDa, dimer 880 kDa) and jack bean urease (monomer 91 kDa, trimer 272 kDa, hexamer 545 kDa) were used in the first dimension as molecular weight markers. For the immunoblotting analysis of the second dimension gel, the reactivity of the M6P/IGF2R mAb MEM-238 and the Plg mAb 7Pg was developed by HRP conjugation and chemiluminescence. C, Human serum sample was diluted 1:1 with PBS and fractionated by gel filtration through Superose 6 column. The fractions were analyzed by immunoblotting using the anti-M6P/IGF2R mAb MEM-238, anti-Plg mAb 7Pg, and rabbit polyclonal antiplasmin Ab. Molecular weight standards are specified in Methods. D, Representative fractions 6, 15, and 24 standardized to the same concentration (5 µg/mL) were subjected to an activation assay in wells coated with uPA together with the chromogenic plasmin substrate S-2251 (0.8 mmol/mL). After 6 hours, the absorbance change at 405 nm was monitored. *P<0.05, **P<0.005 as indicated (n=3). In A through D, similar results were obtained, with several NTLC patient serum samples containing high concentrations of sM6P/IGF2R (Online Figure II). E, Plg was coated on wells of a 96-well plate. The wells were then incubated with purified sM6P/IGF2R (40 µg/mL) in the absence or presence of M1P, M6P, LTGFβ, IGF2, retinoic acid (RA), peptide 18-36 (pep18-36) or scrambled peptide (pepSCR). Bound material was analyzed as in A.
analyzed both sM6P/IGF2R and peptide 18-36 for their ability to influence Plg function.

We found that sM6P/IGF2R blocked the binding of Plg to the cell surface similarly to the lysine analog tranexamic acid (TA), an inhibitor of lysine-dependent binding of Plg to its receptors, by \( \approx 60\% \). Peptide 18-36 blocked the cell surface binding by 45% compared to scrambled peptide (Figure 3A). Because it had been shown before that a direct interaction between uPA and Plg was necessary for Plg activation on the cell surface,\(^{30,31}\) we tested whether sM6P/IGF2R and peptide 18-36 were able to block this interaction. An in vitro binding assay revealed that similarly to TA, sM6P/IGF2R and peptide 18-36 both blocked the interaction between Plg and uPA (Figure 3B). The efficient blocking concentration for sM6P/IGF2R was 40 \( \mu \)g/mL (\( \approx 200 \) nmol/L); for peptide 18-36 it was 2.5 \( \mu \)g/mL (\( \approx 1 \) \( \mu \)mol/L). In parallel, we performed a Plg activation assay in a cell-free system on uPA-coated plastic wells. We found that both sM6P/IGF2R and peptide 18-36 inhibited Plg activation (Figure 3C). In contrast to the Plg activation assay, in the binding assay peptide 18-36 blocked at the lower concentration (compare Figure 3B and 3C), which could be attributable to the higher stringency condition in the binding assay. These data suggest that sM6P/IGF2R exerts its negative regulatory functions in Plg activation via preventing Plg from its binding to uPA, and peptide 18-36, derived from the Plg-binding site of M6P/IGF2R, can mimic sM6P/IGF2R in this activity.

**Modulation of Angiogenesis and Tumor Growth by sM6P/IGF2R and the M6P/IGF2R-Derived Peptide 18-36**

Given that both endothelial and tumor cells use the uPA/Plg system to invade tissues during angiogenesis\(^6\) and tumor pro-

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**Figure 3. sM6P/IGF2R blocks Plg activation.**

**A,** Plg (20 \( \mu \)g/mL) was preincubated for 30 minutes on ice with or without tranexamic acid (10 mmol/L), peptide 18-36 (pep18-36) (10 \( \mu \)g/mL), scrambled peptide (pepSCR) (10 \( \mu \)g/mL), or purified sM6P/IGF2R (40 \( \mu \)g/mL). In parallel, HUVECs were detached with EDTA (no trypsin), washed, and then incubated for 30 minutes on ice with the Plg preparations. Afterward, the cells were washed and cell surface-bound Plg was analyzed by flow cytometry by using the anti-Plg mAb 4Pg or an isotype control mAb (ctr) followed by a FITC-conjugated anti-mouse Ab. Histograms of a typical experiment are shown. **B,** uPA was coated on a 96-well plate and incubated 4 hours on ice with purified Plg in the absence or presence of the indicated peptides (2.5, 5, 10, and 20 \( \mu \)g/mL), purified sM6P/IGF2R (5, 10, 20, and 40 \( \mu \)g/mL), or tranexamic acid (5 mmol/L). Bound material was analyzed by immuno blotting (IB) using anti-Plg antibody 7Pg. **C,** Wells of a 96-well plate were first coated with uPA and then incubated with Plg in combination with the chromogenic plasmin substrate S-2251 (0.8 mmol/mL) at 37°C. Before adding Plg, they were preincubated for 30 minutes on ice with purified sM6P/IGF2R (5, 10, 20, and 40 \( \mu \)g/mL), the indicated peptides (5, 10, 20, and 40 \( \mu \)g/mL), tranexamic acid (5 mmol/L), or aprotinin (5 \( \mu \)g/mL). After 2 hours, the absorbance change at 405 nm was monitored. \( P<0.05, \) \( **P<0.005 \) as indicated (n=3).
gression.2 we tested whether sM6P/IGF2R as well as the sM6P/IGF2R-mimicking peptide 18-36 could modulate these processes.

First, we tested sM6P/IGF2R and peptide 18-36 in an angiogenesis assay in vitro. We seeded primary HUVECs on Matrigel, whereon they form a tube-like network. The total tube length of the cells grown on Matrigel preincubated with peptide 18-36 at the final concentration of 20 μg/mL was reduced by 61% (P=1×10⁻⁴). A similar effect we observed with the cells treated with sM6P/IGF2R (46% and 67% reduction with 20 and 40 μg/mL, respectively), in contrast to the cells incubated without these substances or scrambled peptide. Notably, aprotinin, MMP-2/MMP-9 inhibitor, or the TACE inhibitor TAPI blocked the tube network formation (Figure 4A and 4B). Next, we performed a tube formation assay with TACE-silenced HUVECs in the presence or absence of sM6P/IGF2R and peptide 18-36. The siRNA-mediated silencing of TACE resulted in a reduction of the total tube length by ~30% when compared to control cells, which is in line with published data.32,33 Addition of sM6P/IGF2R or peptide 18-36 to TACE-silenced cells resulted in a further inhibition by 27% and 37%, respectively; similarly to control cells (28% and 53%, respectively; Online Figure IV, A and B). We also tested immortalized HUVECtert with and without silenced expression of M6P/IGF2R8 for their ability to form tubes in the same assay. In comparison to the primary HUVECs, HUVECtert formed a less developed tube-like network that might be attributed to the higher expression of M6P/IGF2R on the surface of these immortalized cells (data not shown). However, on M6P/IGF2R silencing the tube formation was forwarded by 35% (P=0.04), again supporting the regulatory role of M6P/IGF2R in angiogenesis (Online Figure IV, C and D).

The strong antiangiogenic effect of peptide 18-36 in the in vitro tube formation assay suggested a pharmacological potential of this substance. Therefore we tested peptide 18-36 further in in vivo models in mice.

First, we generated mouse analogues of peptide 18-36 (peptide 22-40m); the corresponding sequence is highly conserved between the species (Online Figure V). Next, we analyzed its ability to inhibit the formation of vessel-like structures in an in vivo angiogenesis model by using a murine Matrigel plug assay. Peptide 22-40m or control peptide was mixed into Matrigel solution in the presence of VEGF (10 nmol/L). These mixtures were injected subcutaneously into mice and analyzed 5 days thereafter. In the presence of scrambled peptide, CD31⁺ endothelial cells massively invaded the plugs and formed vessel-like structures (Figure 4C). In contrast, when we mixed peptide 22-40m into Matrigel, we observed significantly reduced endothelial cell numbers within the plugs (64% reduction; P=8×10⁻⁶) and nearly no vessel-like structures. Instead, CD31⁺ endothelial cells stayed confined on the rim of the Matrigel (Figure 4C and 4D). Functionality of the vessel-like structures in the control plugs was manifested by the presence of Ter119⁺ erythrocytes, whereas no erythrocytes were detected in the plugs when peptide 22-40m was incorporated (data not shown). Moreover, peptide 22-40m reduced significantly the total cell number in the plugs (40% reduction; P=2.75×10⁻³) indicating that peptide 22-40m affected the invasion of other cells as well. To identify the type of these cells we stained the plugs with mAbs against various markers and found that CD11b⁺ cells were significantly reduced in the plugs with peptide 22-40m (44% reduction, P=2.70×10⁻⁴). In contrast, we did not observe significant differences with markers for granulocytes, monocytes and macrophages (Gr-1, MoMa-2 and F4–80) (Figure 4D). In addition, we did not detect positive cells within the plugs by using mAbs against CD3 (the marker for T cells), CD45RB (B cells) and NK1.1 (natural killer cells). Based on these findings, we assume that the CD11b⁺ cells could be a subset of dendritic cells. Strikingly, dendritic cells have been shown to play an important role in regulation of angiogenesis34,35 and, similarly to endothelial cells, do use the uPA/Plg system to migrate.36

Second, we studied the effect of peptide 22-40m on tumor growth in a chimeric in vivo model by growing human melanoma cells in SCID mice: the human melanoma M24met cells were injected into the right flank of an animal and at the same time an osmotic pump filled with either scrambled peptide or peptide 18-36 was implanted at a distal position in the same flank. Tumor size was measured daily. After 14 days, peptide 18-36 inhibited significantly the tumor growth (~50% reduction; P=0.02; n=4; Figure 4E). Then we went back to a pure human system and tested the potential modulatory role of peptide 18-36 on cell invasion in a tumor cell invasion model based on basement membrane and Matrigel in a Boyden chamber set-up. Peptide 18-36 significantly inhibited FCS/EGF-induced invasion of the human kidney carcinoma TCL-598 cells (Figure 4F). Importantly, peptide 18-36 inhibited cell invasion to the same extent as the broad MMP inhibitor galardin (GM6001) or the uPA inhibitor aminolide. The relative effects of the peptide on tumor endothelial cells versus tumor cells in vivo remain to be investigated in future.

Discussion

M6P/IGF2R is expressed ubiquitously in a majority of higher eukaryotic cell types. Cellular M6P/IGF2R is known to possess variated binding capacity and be involved in manifold physiological functions.37,38 The physiological role of its soluble form has not been clearly resolved yet. A task in transporting and neutralization of IGF2 was suggested.39–41 However, a reduction of organ size by sM6P/IGF2R was observed also independent of the IGF2 neutralization.42 Furthermore, a role in transporting lysosomal enzymes was discussed.43 In addition, M6P/IGF2R is known to bind a variety of ligands present in serum such as LTGFβ,44 heparanase,35 leukemia inhibitory factor,46 or Plg.7 Based on the results presented here, we suggest that sM6P/IGF2R inhibits Plg activation through preventing Plg from binding to uPA and in this manner modulates cell invasion. This regulatory mechanism of sM6P/IGF2R seems to be different from the inhibitory effect mediated by plasminogen activator inhibitor (PAI)-1, which involves internalization of the uPA-uPAR complex.47 The difference is indicated by several data. First, sM6P/IGF2R and PAI-1 exhibit additive inhibitory effects on Plg activation (Online Figure VI). Second, PAI-1 inhibited Plg activation even in the absence of cellular M6P/IGF2R.7,8
Figure 4. M6P/IGF2R-derived peptide 18-36 inhibits endothelial tube formation, cell invasion, and tumor growth. A, In vitro formation of capillary tube-like structures by primary HUVECs was analyzed in a 96-well tissue culture plate in Matrigel matrix. After polymerization, the Matrigel was preincubated either with purified sM6P/IGF2R (20 or 40 μg/ml), the indicated peptides (20 μg/ml), or protease inhibitors (aprotinin [10 μg/ml], MMP-2/MMP-9 inhibitor I [M2/9i] [10 μmol/L], TAPI [10 μmol/L]) in M-199 medium for 30 minutes at 37°C or were left untreated. Then, the cells (5×10^4 cells/well) were seeded on the Matrigel and incubated for 18 hours at 37°C. Images were taken by using a phase-contrast microscope. B, Cell tube length observed in A was analyzed in 4 random fields of 2 independent wells. *P<0.05, **P<0.005 as indicated (n=4). C, In vivo angiogenesis murine Matrigel plug assay: Matrigel plugs (600 μL) supplemented with VEGF (10 nmol/L) and the indicated peptides (20 μg/ml) were injected subcutaneously. After 5 days, mice were euthanized and the Matrigel plugs were removed, fixed in 4% paraformaldehyde, embedded in paraffin, and stained. The samples were analyzed with an Olympus AX70 microscope. Digital images were taken by an F-view camera. D, Numbers of cells in Matrigel plugs from C were quantified on images displaying sections of the complete plugs. The images were divided into 1-mm² segments, and the number of cells within each segment was counted. Data from a representative experiment are shown. Similar results were obtained.
Third, neither cellular M6P/IGF2R nor a peptide derived from M6P/IGF2R affected uPAR internalization.4,8

The release of M6P/IGF2R from the cell, detected in the past, seemed to occur by cell surface proteolysis9,11,16,48; however, an alternative nonproteolytic pathway was proposed as well.12 Here we show that TACE/ADAM-17 mediates shedding of M6P/IGF2R; though our data do not exclude contributions of other mechanisms. ADAM family proteases have crucial roles in angiogenesis and cancer biology49; in particular, TACE was suggested to be proangiogenic through MMP-2 activation12 and also to be involved in pathological neovascularization.33 Our data indicate that TACE contributes also to a negative regulation of angiogenesis through the release of M6P/IGF2R that downregulates Plg activation. Interestingly, plasmin itself can contribute to the activation of MMP-2.9 Because TACE can cleave a wide array of plasma membrane proteins,30,51 it is reasonable to speculate that the TACE-mediated regulation of angiogenesis depends on the receptor milieu. Whether TACE-mediated shedding is the major source of sM6P/IGF2R in vivo and how this mechanism contributes to the regulation of angiogenesis at the physiological or pathological level should be clarified in future studies. Because a conventional whole body knockout of TACE is neonatally lethal,12 inducible conditional knockouts will be highly useful for these studies.

By BN-PAGE and gel filtration we found sM6P/IGF2R to be complexed with a fraction of Plg Plg is present in serum at a concentration of ~2 μmol/L.18 We detected different serum sM6P/IGF2R-Plg complexes. Namely, we found Plg and sM6P/IGF2R in an apparently high-molecular weight complex and also a complex of intermediate molecular weight. On the other hand, the majority of Plg was present in smaller forms corresponding to the size of the single Plg molecule (Figure 2). A precise composition and functionality of these complexes need to be solved in future. Interestingly, the binding of Plg to the plasma histidine-rich glycoprotein was found to enhance its activation51; in contrast, our data indicate that the serum Plg complexed with sM6P/IGF2R is less responsive to the activation by uPA than the single Plg molecule.

sM6P/IGF2R appears to block Plg activation at a concentration of 200 nmol/L (Figure 3), i.e., with similar affinity as M6P/IGF2R binds to Plg.21,23 In healthy donors, we measured the concentration of sM6P/IGF2R to be 6 μg/mL (± 1 μg/mL; n = 6) corresponding to ~27 nmol/L (Online Figure II), which was to some extent higher than the 3 to 4 nmol/L reported previously for healthy adult humans.16 This difference could be caused by different capabilities of various mAbs to bind and detect differentially truncated sM6P/IGF2R. In fact, the values of sM6P/IGF2R measured by specific immunoassays might be underrated because they most likely related to the whole ectodomain. Shorter fragments, which were also detected in serum (Figure 2A),10,28 could escape a standard ELISA- or Western blotting-based measurement by the lack of antibody recognition. Such fragments could be produced by proteolytic degradation, for example mediated by plasmin (Online Figure III), and may presumably have similar functional properties as sM6P/IGF2R, on the condition that they still retain the Plg-binding region. This is reasonable because plasmin has a natural affinity to cleave proteins at lysine residues54 and there are several lysines within the amino-terminal Plg-binding part of sM6P/IGF2R (Online Figure V). The processing of Plg receptors by plasmin is a well-characterized pathway to upregulate Plg binding to cells via C-terminal lysines.29 Therefore, one can speculate that not only new Plg-binding sites are generated on the cell surface in this way but also soluble peptides capable of modulating Plg function.

The serum concentration of sM6P/IGF2R may be regulated developmentally or altered during certain disorders.16 We show here that the serum concentration of sM6P/IGF2R dramatically increases in liver cirrhosis patients (Online Figure II). It is known that there are many changes in the fibrinolytic system accompanying severe hepatic disease, which may affects fibrinolytic balance.55,56 Whether the increased level of sM6P/IGF2R is associated with the increased tendency to develop thromboses in patients with cirrhosis must be evaluated in future. High concentrations of sM6P/IGF2R might be reached in certain environmental conditions such as cell-cell or cell-matrix contact zones in the bargain. The interaction of sM6P/IGF2R with Plg may be of considerable regulatory importance within the microenvironment of migrating cells in which the uPA/uPAR complex is localized in leading edges8 to activate cell-surface Plg.30,31 One could also speculate that secreted sM6P/IGF2R may through its properties contribute to tumor-suppressive senescence mechanisms attributed to M6P/IGF2R.57

Finally, peptide 18-36 that is derived from the Plg binding site within the amino-terminal region of M6P/IGF2R7,24 does possess a modulatory capability in angiogenesis and tumor growth (Figure 4). This property of peptide 18-36 may result from its inhibitory effect on uPA-Plg binding and Plg activation (Figure 3). Thus, the migration and growth of cell types harnessing the Plg system should be specifically blocked by peptide 18-36, as actually demonstrated for endothelial cells, CD11b+ cells (putative dendritic cells), and

![Figure 4](http://circres.ahajournals.org/)

Figure 4 (Continued). with 4 other pairs of Matrigel plugs. The number of endothelial cells was measured by staining with a CD31 mAb, and the total cell number was evaluated by DAPI staining. In addition, granulocytes were evaluated by staining with mAb Gr-1, monocytes by mAbs MoMoa-2 and F4–80, CD11b+ cells (monocytes, macrophages, dendritic cells) by CD11b mAb. *P<0.05, **P<0.005, ***P<0.0005 as indicated (mean±SD; n=14). E, To study the effect of peptide 18-36 on tumor growth in vivo, the human melanoma M24met cells were injected into the right flank of pathogen-free 6-week-old female CB17 SCID mice. At the same time, osmotic pumps filled with either scrambled peptide or peptide 18-36 were implanted at a distal position in the same flank. Tumor size was measured daily during the 15 days of treatment. *P<0.05, **P<0.005 as indicated (mean±SEM; n=4). F, Effect of peptide 18-36 on tumor cell invasion was tested by using a modified Boyden chamber coated with basement membrane and Matrigel. The Matrigel was preincubated with the peptides (20 μg/mL) or the indicated inhibitors in FCS-free RPMI-1640 for 1 hour at 37°C. Then, human kidney carcinoma TCL-598 cells were seeded on top of the filter and incubated for 18 hours at 37°C. EGF (10 ng/mL) in RPMI-1640 plus 10% FCS was used as chemoattractant in the bottom chamber. The concentrations of the used inhibitors were: galardin/GM6001 (GM) (10 μmol/L), amiloride (10 μmol/L), pepstatin A (10 μmol/L), and E-64 (10 μmol/L). Results are representative for 3 independent experiments. *P<0.05, **P<0.005 as indicated (n=3).
tumor cells (Figure 4). Using this peptide for pharmacological downregulation of the Plg activation system may represent a novel therapeutic approach that would inhibit tumor-associated angiogenesis and tumor progression.

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Disclosures
None.

References


42. Lorenzo K, Ton P, Clark JL, Coulibaly S, Mach L. Invasive properties of malignant cells hijack them for invasion and metastasis.

What New Information Does This Article Contribute?

Cell movement is required for several physiological processes; however, rampant cell migration is a hallmark of tumor cell dissemination and tumor-associated angiogenesis. Cell migration engages several processes including cell adhesion, pericellular proteolysis, and cytoskeletal rearrangements. The cell-associated uPA system results in the generation of the potent serine protease plasmin and is therefore essential for the proteolytic breakdown of extracellular matrix proteins within tissue barriers. Thus, the uPA system plays a key role in tumor progression and specific targeting of this system has been recognized as a promising approach to treat carcinogenesis. Here, we show that sM6P/IGF2R inhibits pericellular Plg activation by blocking the interaction between Plg and uPA. In view of these observations it is possible that elevated concentrations of sM6P/IGF2R in sera of liver cirrhosis patients might contribute to deregulation of fibrinolysis accompanying this disorder. Our observation that the release of sM6P/IGF2R is mediated by TACE adds a new facet to the role of this sheddase in regulating angiogenesis and tumor progression. Our results showing that peptide 18-36 derived from the Plg-binding site of M6P/IGF2R mimics sM6P/IGF2R in the inhibition of Plg activation and blocks cancer cell invasion in vitro, endothelial cells invasion in vivo and tumor growth in vivo.

Novelty and Significance

What Is Known?

- Pericellular proteolytic systems, such as the urokinase plasminogen activator (uPA)/plasmin system, are essential for cells that have to migrate to execute their functions (for instance, immune cells to target pathogens or endothelial cells to form vessels). However, malignant cells hijack them for invasion and metastasis.

What New Information Does This Article Contribute?

- TACE mediates the release of the ectodomain of M6P/IGF2R (soluble M6P/IGF2R) from human endothelial cells.
- The levels of sM6P/IGF2R are elevated in sera of liver disease patients.
- sM6P/IGF2R binds plasminogen (Plg) and thereby prevents Plg from binding to the cell surface and uPA, ultimately inhibiting in this manner Plg activation to active plasmin.
- Peptide 18-36 derived from the Plg-binding site of M6P/IGF2R mimics sM6P/IGF2R in the inhibition of Plg activation, and blocks cancer cell invasion in vitro, endothelial cells invasion in vivo and tumor growth in vivo.
Soluble M6P/IGF2R Released by TACE Controls Angiogenesis via Blocking Plasminogen Activation

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Expanded Supplemental Materials and Methods

Antibodies

The monoclonal antibodies (mAbs) MEM-238 and MEM-240 to M6P/IGF2R were generated by us. MEM-238 was produced and purchased from EXBIO (Prague, Czech Republic); MEM-240 and MEM-M6/1 (CD147) was produced and provided by Dr. Václav Hořejší (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague). The mAb H2 to uPAR was provided by Dr. Ulrich Weidle (Roche Diagnostics, Division Pharma, Penzberg, Germany). The goat polyclonal Ab to M6P/IGF2R was kindly provided by Dr. Stefan Höning (Center for Molecular Medicine, University of Cologne, Germany). The rabbit polyclonal Ab Ab2051 to TACE, rabbit polyclonal Ab to alpha 5 integrin and rabbit polyclonal anti-plasmin Ab were from Abcam (Cambridge, UK). Anti-MMP8 mAb was from Calbiochem. The mAbs against Plg (4Pg, 7Pg) were from Technoclone (Vienna, Austria). The anti-goat IgG Ab-HRP conjugate was obtained from R&D Systems and the anti-rabbit and anti-mouse IgG Ab-HRP conjugates from Sigma-Aldrich. The streptavidine-HRP conjugate was supplied by GE Healthcare (Uppsala, Sweden). The mAbs against various differentiation markers used in the Matrigel plug assay were purchased either from eBioscience (Hatfield, UK): Gr-1 (a marker for granulocytes), Ter119 (erythrocytes), CD11b (macrophages, natural killer cells, granulocytes, activated lymphocytes and B-1 cells), NK1.1 (natural killer cells), CD45RB (peripheral B cells); from AbD Serotec (Düsseldorf, Germany): MoMa-2 and F4-80 (both markers for monocytes and macrophages); or from Becton Dickinson (San Jose, CA): CD3 (T cells) and CD31 (endothelial cells).

Peptides

Peptides derived from the N-terminal part of M6P/IGF2R (human peptide 18-36, TKNNVLYKINICGSVDIVQ; mouse peptide 22-40m, SKNNAVYKINVCGNVGISS) and control scrambled peptide (pepSCR, SVNCAIGSNGKVNLYIKVNS) were produced either by Genosphere (Paris, France); or by Peptide 2.0 Inc. (Chantilly, VA).

Other materials

Endothelial cell growth supplement, human uPA, pro-uPA, PAI-1 and Glu-Plg were products of Technoclone (Vienna, Austria). BSA was from Roth (Karlsruhe, Germany). Tricine, Tris, Coomassie brilliant blue (CBB) (R-250 and G-250), ammonium persulfate, TEMED, sodium dodecyl sulfate (SDS), acrylamide, N,N′-methylenebis-acrylamide were purchased from SERVA (Heidelberg, Germany). Leupeptin (inhibitor of serine and cysteine proteases), pepstatin A (inhibitor of aspartyl proteases), E-64 (inhibitor of cysteine proteases), aprotinin (inhibitor of serine proteases), alpha-2 antiplasmin (inhibitor of plasmin), crystal violet, Triton X-100, human epidermal growth factor (EGF), heparin, mannose 6-phosphate (M6P), mannose 1-phosphate (M1P), retinoic acid (RA) and tranexamic acid (TA) were from Sigma-Aldrich (St. Louis, MO). Nonidet P-40 and EZ-Link Sulfo-NHS-biotin were obtained from Pierce (Rockford, USA). All MMP inhibitors were from Calbiochem (Darmstadt, Germany). Plasmin and plasmin-specific substrates S-2251 were products of CoaChrom Diagnostica (Vienna, Austria). Insulin-like growth factor 2 (IGF2) was from Immuno Tools (Friesoythe, Germany), latent transforming growth factor β (LTGFβ) from R&D Systems (Minneapolis, MN).
Serum samples

Human serum samples were obtained from healthy donors or from patients with various liver diseases after informed consent that a part of the routine blood samples will be used for research purposes (approved by the Ethical Committee of the Medical University of Vienna). All samples were stored at -20°C before use.

Cell culture

Generally, cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine and 10% heat inactivated FCS (Sanova Diagnostics, Vienna, Austria). The human kidney epithelial tumor cell line TCL-598 was a gift from the Novartis Research Institute (Vienna, Austria). All cells were grown in a humidified atmosphere at 37°C and 5% CO2 and passaged twice a week using trypsin-EDTA solution. Primary human umbilical vein endothelial cells (HUVEC) were kindly provided on a regular basis by Renate Hofer-Warbinek (Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria). They were isolated from umbilical cord and cultured up to passage 6 in M199 medium (Sigma-Aldrich) supplemented with growth factors (Technoclone), heparin and 20% FCS as described. The immortalized HUVEC (HUVECTert) generated previously by us were cultured under the same condition as primary HUVEC. The human melanoma cell line M24met was cultivated in IMDM medium supplemented with 10% fetal calf serum (FCS, Life Technologies Inc., Vienna, Austria), 2 mM/L glutamine (Life Technologies Inc.) and 50 IU/ml penicillin-streptomycin (Life Technologies Inc.). The immortalized mouse embryonic fibroblasts (mEF) isolated from E13.5 TACEembryos together with wild type mouse embryonic fibroblasts were generously provided by Dr. Carl. P. Blobel (Hospital for Special Surgery, NY) and were cultivated in DMEM medium in flasks pre-coated with gelatine.

To analyze release of sM6P/IGF2R, HUVEC, HUVECTert or mEF were seeded at a density of 1x10^5/well in a 48-well plate and cultivated until confluence. Then, the medium was exchanged and the cells were cultivated for 24 h under FCS-free condition. Afterwards, the supernatant was taken, cleared by centrifugation and analyzed in parallel to the cell lysate by immunoblotting.

Flow cytometry

Cells were detached using 3 mM EDTA/PBS, washed with PBS containing 1% BSA and 0.02% sodium azide, and afterwards incubated for 30 minutes with specific mAbs. After 30 min. Then the cells were washed again and a second step staining was done with FITC conjugated F(ab')_2 anti-mouse IgG+IgM antibodies (An der Grub, Kaumberg, Austria). Prior to analysis, the cells were washed again. Dead cells were excluded by staining with 7-amino-actinomycin D (7-AAD). Flow cytometry was performed with a LSR II flow cytometer (Becton Dickinson). Data acquisition was executed with the FACS DIVA software. Data analysis was accomplished with the FlowJo software (Treestar Inc., Ashland, OR).

RNA interference

To silence the expression of TACE and MMP-8, we transiently transfected HUVEC with 1 µg siRNA using the siPORT Transfection agent from Ambion according to the manufacturer’s instructions (Austin, TX). Two validated short interfering RNAs (siRNAs) targeting human TACE (target transcript 6868|NM_003183) were from QIAGEN (Hilden, Germany): TACE1 – Hs_ADAM17_7 HP, CAGGATGTAATGAACGATT; and TACE2 – Hs_ADAM17_8 HP, AAGAAACAGAGTGCTAATTTA. One validated siRNA targeting MMP-8 (Hs_MMP8_1 HP, TCCCAAGGATATATCAAACTA) and the Silencer Negative Control siRNA were from QIAGEN too. Assays were generally done 48 hours after transfection.
The generation of immortalized human endothelial cells (HUVECtert) was described previously together with the preparation of these cells with silenced expression of M6P/IGF2R. Briefly, the stable knockdown of M6P/IGF2R was performed by the transduction of a M6P/IGF2R-specific short hairpin RNA (shRNA) expression cassette. As a control construct (shCTR), the MISSION nontarget shRNA control vector (pLKOpuro1; Sigma-Aldrich) was used. Lentiviral particles were generated as described, and 48 hours after transfection viral supernatants were used to transduce HUVECtert. The transduced shM6P/IGF2R- and shCTR HUVECtert cells were cultured with puromycin (2 µg/mL).

Immunoprecipitation and immunoblotting analysis

Human serum samples were diluted 20 times with PBS and subjected to immunoprecipitation using specific mAbs coated on a 96-well BD Falcon™ plastic plate via a goat anti-mouse IgG Ab (Sigma). After 3 h at 4°C, the wells were washed and the immunoprecipitates were analyzed by SDS-PAGE followed by transfer to Immobilon polyvinylidene difluoride membranes (Millipore Co., Bedford, MA). The membranes were blocked using 4% nonfat milk and immunostained directly or indirectly with specific mAbs conjugated with HRP followed by chemiluminescence. For visualization of the proteins, the chemiluminescence image analyzer FUJIFILM/LAS-4000 was used. In other experiments, cell lysates, supernatants, purified proteins or samples from binding assays were also analyzed by SDS-PAGE followed by immunoblotting.

Blue-native electrophoresis

For the first dimension of the two-dimensional Blue Native/SDS-PAGE electrophoresis (BN-PAGE), a native 7.5% gel was used as a separation gel and a native 2.5% gel as a stacking gel. The samples were loaded and deep-blue cathode buffer (50 mmol/L Tricine, 7.5 mmol/L imidazole pH 7.0 and 0.02% Coomassie blue G 250) was poured on top of the samples into the upper (cathode) chamber. The anode buffer (25 mmol/L imidazole-HCl; pH 7.0) was poured into the lower (anode) chamber and electrophoresis was performed at 80 V and room temperature overnight. The following day, after the samples entered the first third of the gel, the deep-blue cathode buffer was replaced with the slightly-blue cathode buffer (50 mmol/L Tricine, 7.5 mmol/L imidazole pH 7.0 and 0.002% Coomassie blue G 250) and the voltage was increased to 200V. Ferritin (monomer 440 kD, dimer 880 kD), jack bean urease (monomer 91 kD, trimer 272 kD, hexamer 545 kD) and BSA (all from Sigma) were used as markers. Lanes were cut from the gel, put on the top of a 7.5% SDS-PAGE gel and equilibrated in running buffer for 45 min. Afterwards the gel was run at 10 V at room temperature overnight. The next day the voltage was increased to 200 V and the gel blotted at constant voltage (19 V) onto a Immobilon polyvinylidene difluoride membrane. The membrane was blocked with 4% milk and then incubated with specific antibodies and visualized.

Protein purification and in vitro binding assay

Human THP-1 cells (5×10⁶/ml) were cultivated in FCS-free RPMI-1640 medium for 24 h. The cells were spun down and sM6P/IGF2R was purified from the supernatant on an affinity chromatography column of anti-M6P/IGF2R mAb MEM-238 coupled to CNBr-activated Sepharose (Pharmacia, Stockholm, Sweden). The eluate was concentrated by the Centrifugal Filter Device Ultracell 30k (Millipore Bioscience Research Reagents, Temecula, CA).

For the binding assay, 5 µg/ml of various molecules were coated on wells of a 96-well BD Falcon™ plate in PBS (pH 8.7) for 2 h at 37°C. Then the wells were blocked with 1% BSA in PBS for 1 h at room temperature and washed two times with binding buffer (20 mmol/L Tris-
HCl, 140 mmol/L NaCl, pH 7.5). Afterwards, the wells were incubated for 4 h on ice with binding buffer supplemented with 5 µg/ml purified assayed proteins in the absence or presence of additional molecules, and washed twice with ice-cold binding buffer. Bound material was analyzed by SDS-PAGE and immunoblotting.

**Gel filtration**

The GE Healthcare's AKTA FPLC System was used for the gel filtration experiments. Samples were loaded at room temperature in a volume of 100 µl onto a Superose 6 HR 10/300 GL column (Pharmacia) equilibrated with PBS. The absorbance at 280 nm was monitored and fractions of 500 µl were collected and analyzed by immunoblotting. The following standards of molecular weight (all from Pharmacia or Sigma) were used: blue dextran (2.000 kD), thyroglobulin (609 kD), ferritin (440 kD), catalase (232 kD), aldolase (168 kD), BSA (60 kD), and carbonic anhydrase (29 kD).

**Enzyme-linked immunosorbent assay (ELISA)**

The mAb MEM-240 to M6P/IGF2R was coated on a 96-well immunoplate (Nunc, Glostrup, Denmark) in carbonate buffer (pH 9.6; 500 ng/well) by an overnight incubation at 4°C. The plates were washed and blocked with 1% BSA in PBS. The wells were then incubated for 2 h at room temperature either with serum samples (200 x diluted in PBS) or with standard samples (purified M6P/IGF2R). Afterwards, the plates were washed and incubated for 2 h at room temperature with the biotinylated anti-M6P/IGF2R mAb MEM-238 to detect sM6P/IGF2R. mAb MEM-238 was biotinylated by using EZ-Link Sulfo-NHS-biotin according to the manufacturer's instructions. After washing, the plates were incubated with streptavidin AP-conjugate (Chemicon, Millipore) for 45 min at room temperature followed by incubation with the PNPP substrate (Sigma) for 30 min at room temperature. The reaction was stopped by the addition of 3 mol/L NaOH and the absorbance was measured at 405 nm using an enzyme-linked immunosorbent assay reader (Anthos Labtec Instruments, Salzburg, Austria).

**Plasmin and uPA activity assay**

In a cell-free system, uPA (10 nmol/L) was coated on a 96-well BD Falcon™ plate in PBS (pH 8.7) for 2 h at 37°C. The wells were blocked with 1% BSA in PBS for 1 h at room temperature, washed two times with PBS and incubated at 37°C with human Glu-Plg (50 nmol/L) in PBS. Both uPA and plasmin activities were analyzed by adding either the chromogenic plasmin (S-2251) or uPA (S-2444) substrate (both 0.8 mmol/mL). The absorbance change at 405 nm was monitored at different time points by using a 96-well plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). In order to block the activity of unbound plasmin, the assays were performed in the presence of alpha-2 antiplasmin (2.5 µg/mL). Optionally, various inhibitors were added to the reaction.

In a cell system, HUVEC (1x10^5 cells/well) were seeded in 96-well plates 24 h before the assay. Cell monolayers were washed and incubated with pro-uPA (3 nmol/L) at 37°C for 20 min. After washing, Plg (50 nmol/L), the chromogenic plasmin substrate S-2251 (0.8 mmol/mL), alpha-2 antiplasmin (2.5 µg/mL) and optionally PAI-1 (10 U/mL) and/or sM6P/IGF2R were added. Cells were incubated at 37°C and after 5 hours the absorbance change at 405 nm was monitored as described above. The whole experiment was done in serum-free medium.

**Cell invasion assay**

Cell invasion was measured with the Chemicon QCM™ invasion assay kit according to the manufacturer’s instructions (Millipore). Briefly, the invasion assay was based on the Boyden
chamber principle with filter inserts containing 8 µm pore size polycarbonate membranes coated with a basement membrane and Matrigel matrix. Optionally, the Matrigel was pre-incubated with inhibitors or peptides diluted in FCS-free RPMI-1640 medium for 1 h at 37°C. EGF in RPMI-1640 medium supplemented with 10% FCS was added as chemoattractant into the lower chambers (10 ng/mL). Then, the cells were suspended in RPMI-1640 medium and seeded into the upper part of the filter (1x10^5 cells/filter) and incubated for 18 h at 37°C. The number of cells that invaded onto the lower side of the filters was evaluated after staining with crystal violet either by cell counting or by lysing the crystal violet-stained cells followed by measuring the absorbance (OD595; corresponding to the relative cell number) with a plate reader (SpectraMax M5, Molecular Devices).

Endothelial cell tube formation assay

The in vitro endothelial cell tube formation assay was done as described previously9. Matrigel (BD, Franklin Lakes, NJ) was added (50 µl) to wells of a 96-well tissue culture plate and allowed to polymerize for 30 min at 37°C. Afterwards, the Matrigel was optionally pre-incubated with peptides in M199 medium (25 µL) for 30 min at 37°C. HUVEC or HUVECtert were re-suspended in M199 medium and added to the plate (5x10^4 cells/well), optionally with the indicated compound. The cells were incubated for 18 hours at 37°C and tube formation was analyzed by light microscopy. Images were taken with a phase-contrast microscope (Nikon Diaphot TMD, Nikon, Tokyo, Japan) using a cooled charge coupled device camera (Kappa DX30, Kappa GmbH, Gleichen, Germany). Four random fields were chosen in two independent wells and the tube-length was analyzed by using the Lucia software (Laboratory Imaging Ltd., Nikon, Champigny-Sur-Marne, France). Notably, we did not add Plg because we detected a considerable concentration of this zymogen in the Matrigel (data not shown), which is in accordance with a previous report10.

In vivo Matrigel plug assay

The in vivo angiogenesis Matrigel plug assay was done as described previously11. Briefly, 6-week-old male C57BL/6 mice were used. Matrigel plugs (600 µL) were injected subcutaneously into the flank region together with VEGF (10 nmol/L) and peptides (20 µg/mL). After 5 days, mice were sacrificed and the Matrigel plugs were removed, fixed in 4% paraformaldehyde, embedded in paraffin and stained with different marker mAbs. The samples were analyzed with an Olympus AX70 microscope (Olympus Optical Co., Tokyo, Japan). Digital images were taken by an F-view digital camera (Soft Imaging Systems, Münster, Germany). The numbers of cells in the Matrigel plugs were quantified on pictures displaying sections of the complete plug. The pictures were divided into 1 mm² segments, and the number of cells within all segments was counted. Five different Matrigel plugs were screened for each peptide treatment. Cell numbers were evaluated either by the AnalySIS Pro software system (Soft Imaging Systems) or by the Lucia software (Laboratory Imaging Ltd., Nikon).

SCID mouse tumor model

All procedures were carried out in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines and Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, National Institutes of Health, Publication No. 86-23). In addition, all experiments were approved by the ethics committee of the Medical University of Vienna and by the Austrian Government Committee on animal experimentation.

Pathogen-free 6 week old female CB17 scid/scid (SCID) mice (Charles River, Sulzfeld, Germany) were housed and used as described12. Animals were anesthetized and 1.5x10^6
M24 met melanoma cells were injected into the right flank of the animals. At the same time, Alzet® osmotic pumps #1002 (Durect corporation, Cupertino, CA) filled with an appropriate peptide in PBS were implanted at a distal position in the same flank. The tumor size was measured daily over the 15 days of treatment. The animals were then sacrificed; primary tumors, lymph nodes and lungs were removed, fixed with 4% paraformaldehyde and embedded in paraffin.

**Statistical analysis**

All experiments were performed at least three times in at least triplicates. The data were expressed as mean values with standard deviation. Statistical significance was evaluated by using a Student's t-test; a value of $p^*<0.05$, $p^{**}<0.005$ or $p^{***}<0.0005$ (as indicated) was considered to be significant or highly significant, respectively.
Supplemental Online Figure I.

Analysis of shedding of M6P/IGF2R with control- and M6P/IGF2R-silenced HUVECTert.

To confirm the specificity of the observed band of 220 kD in the supernatant of HUVEC, we checked the supernatants derived from immortalized human endothelial cells (HUVECTert) generated previously by us. When we silenced the expression of M6P/IGF2R with a short hairpin RNA specific for shM6P/IGF2R (A) the 220 kD band disappeared in comparison to cells transduced with an irrelevant control, shCTR (B). To exclude the possibility that the band simply reflected the presence of floating cells or detritus from lysed cells, we checked the supernatants through Western blotting with CD147 mAb MEM-M6/1 since we had detected the CD147 molecule on the surface of HUVECTert (data not shown). We did not detect any CD147 molecule in the supernatants of the HUVECTert.

After the indicated time intervals, samples were taken from the supernatants for immunoblotting analysis (IB) by using a 10% SDS-PAGE gel. The anti-M6P/IGF2R mAbs MEM-238 and MEM-240 and the CD147 mAb MEM-M6/1, and chemiluminescence were used for development of the blot.
Supplemental Online Figure II.

**Increased concentration of sM6P/IGF2R in sera of liver cirrhosis patients measured by ELISA.**

We generated a sandwich ELISA-based method and measured the concentrations of sM6P/IGF2R in sera from patients with various liver disorders: nutritional-toxic liver cirrhosis (NTLC), primary biliary cirrhosis (PBC), chronic hepatitis type C (HCC) and autoimmune hepatitis (AH). The mAb MEM-240 was used for binding and the biotinylated mAb MEM-238 for detection. Both these mAbs recognize distinct epitopes within sM6P/IGF2R\(^1\), and thus are appropriate for the ELISA-based evaluation. We found in all types of liver disorders higher serum levels of sM6P/IGF2R. However, the highest level, a nearly 6-fold increase compared to the levels in healthy donors’ sera (CTR; p=1.3x10\(^{-3}\); n=10) corresponding to 10 – 60 µg/ml we found in sera of NTLC patients. These sera were used for further analysis.
Supplemental Online Figure III.

**M6P/IGF2R is processed by plasmin.**

Purified M6P/IGF2R (50 µg/mL) was subjected to proteolysis for 15 min at 37°C with the following enzymes: uPA (50 nmol/L), Plg (50 nmol/L), uPA+Plg (both 50 nmol/L) and plasmin (Plm; 10, 20, 50, 100 nmol/L). Treatment was stopped by the protease inhibitor aprotinin (5 µg/mL) and the samples were analyzed by immunoblotting with the polyclonal goat anti-M6P/IGF2R Ab.
Supplemental Online Figure IV.

Tube formation of HUVECtert upon M6P/IGF2R and TACE silencing in an *in vitro* Matrigel matrix assay.

(A) *In vitro* formation of capillary tube-like structures by TACE-silenced HUVEC. TACE-silenced HUVEC (siTACE) and control silenced cells (siCTR) were generated as described in Methods and Figure 1 by using the siRNA construct TACE2 and a control siRNA. Forty-eight hours after transfection, a Matrigel™ matrix was prepared in a 96-well tissue culture plate. After polymerization, the cells were seeded on the Matrigel (5x10⁴ cells/well) and incubated for 18 hours at 37°C. Images were taken by using a phase-contrast microscope. (B) The tube-length in (A) was analyzed in four random fields of two independent wells; p*<0.05, p**<0.005 as indicated (n=4). (C) *In vitro* formation of capillary tube-like structures by HUVECtert silenced for M6P/IGF2R. Cells transduced with a control shRNA (shCTR) or an shRNA specific for M6P/IGF2R (shM6P/IGF2R) were seeded on the Matrigel (5x10⁴ cells/well) and incubated for 18 hours at 37°C as in (A). (D) The tube-length from (C) was analyzed in four random fields of two independent wells; p*<0.05 as indicated (n=4).
Supplemental Online Figure V.

Protein sequence comparison of the amino-terminal regions of the human and mouse M6P/IGF2R.

The sequence of human M6P/IGF2R starts with the residue No. 1 after the signal sequence. Identical residues are highlighted in dark grey, conservative residues in light grey. The sequences of the derived peptides pep18-36 and pep22-40m respectively, are shown.

Human M6P/IGF2R: TKNVLYKINICGNVDIVQG
Mouse M6P/IGF2R: KAVDLYKINICGNVDIVQG

Peptide pep18-36: TKNVLYKINICGSVDIVQG
Peptide pep22-40m: KHNAYKINICGNVDIYSG
Supplemental Online Figure VI.

**The effects of sM6P/IGF2R and PAI-1 on Plg activation.**

HUVEC (1x10^5 cells/well) were seeded in 96-well plates. Cell monolayers were first incubated with pro-uPA (3 nmol/L) at 37° C for 20 min. After washing, Plg (50 nmol/L), the chromogenic plasmin substrate S-2251 (0.8 mmol/mL), alpha-2 antiplasmin (2.5 µg/mL), and optionally PAI-1 (10 U/mL) and/or purified sM6P/IGF2R (20 µg/mL), were added to the reaction. Cells were incubated at 37° C and after 5 h the absorbance change at 405 nm was monitored by using an ELISA reader. The whole experiment was done in serum-free medium. The experiment was performed in triplicates and results are representative for three independent experiments.
Supplemental Online References


