Generation of a novel proteolysis resistant vascular endothelial growth factor$_{165}$ variant by a site-directed mutation at the plasmin sensitive cleavage site

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Abstract Vascular endothelial growth factor (VEGF) is a potent angiogenic mediator in tissue repair. In non-healing human wounds plasmin cleaves and inactivates VEGF$_{165}$. In the present study, we generated recombinant VEGF$_{165}$ mutants resistant to plasmin proteolysis. Substitution of Arg110 with Ala110 or Gln110, and Ala111 with Pro111 yielded plasmin-resistant and biologically active VEGF$_{165}$ mutants. In addition, substitution of Ala111 with Pro111 resulted in a substantial degree of stabilization when incubated in wound fluid obtained from non-healing wounds. These results suggest that the plasmin cleavage site Arg110/Ala111 and the carboxyl-terminal domain play an important role in the mitogenic activity of VEGF$_{165}$.

Key words: VEGF; Protease sensitivity; Plasmin; Mutation

1. Introduction

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific multifunctional cytokine that is a key regulator in physiologic and pathologic processes of angiogenic remodeling (reviewed in [1]). By differential mRNA splicing, the single human VEGF gene gives rise to at least six protein isoforms VEGF$_{121, 145, 165, 183, 189}$, and $_{206}$ [2,3]. Among them, the 165-amino-acid isoform is the major gene product found in human tissues. The splice variants differ primarily in the presence or the absence of the heparin-binding domains encoded by exons 6 and 7, giving rise to forms that differ in their heparin/heparan-sulfate binding ability, as well as their affinities to VEGF receptors flt-1, flk-1/KDR and neuropilin-1 [4–9]. Whereas VEGF$_{121}$ does not bind heparan-sulfate and is freely diffusible, VEGF$_{189}$ binds heparin and is primarily associated with the cell surface and extracellular matrix, and VEGF$_{165}$ has intermediate properties [5,10,11]. Further, native VEGF$_{189}$ binds to flt-1 but not flk-1/KDR [12]. Native VEGF$_{189}$ requires maturation by urokinase (uPA) within the exon 6 encoded sequence to bind to flk-1/KDR and to exert a mitogenic effect on endothelial cells [12]. In contrast, plasmin digestion of VEGF$_{165}$ decreases its mitogenic activity for endothelial cells [13]. Plasmin digestion of VEGF$_{165}$ yields two fragments: an amino-terminal homodimer (VEGF1-110) containing the flt-1 and the flk-1/KDR receptor binding determinants encoded by exons 3 and 4, respectively, and a carboxyl-terminal polypeptide comprising the neuropilin-1 binding site encoded by exon 7 (VEGF111-165) [13]. Interestingly, the reduced mitogenic activity of the amino-terminal homodimer VEGF1-110 is similar to that observed for VEGF$_{121}$ [12,13]. These findings suggest that differential protease susceptibility, extracellular localization and/or receptor binding may result in distinct functions for different VEGF isoforms.

Recently, we demonstrated that the proteolysis of VEGF$_{165}$ by plasmin is increased in wound fluid collected from chronic non-healing wounds versus healing wounds [14]. VEGF plays a critical role during the angiogenic response in tissue repair [15–17], suggesting that VEGF degradation and loss of its biological activity may contribute to an impaired wound healing response. These results prompted us to introduce amino acid alterations at the plasmin sensitive cleavage site of VEGF$_{165}$ (Arg110/Ala111), in order to stabilize the VEGF$_{165}$ molecule. Substitutions at either site result in VEGF$_{165}$ products that, while maintaining growth-promoting properties, are either fully or partially plasmin-resistant. This type of modification would be expected to increase the period that topically applied VEGF protein is active in the wound environment, implying a potential clinical application.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutagenesis was performed using base mismatched oligonucleotides as indicated: Mut$_{Ala110}$ 5′-GACCAAAGAAAGAT**GC**CGCAAGACAAG-3′, Mut$_{Gln110}$ 5′-GACCAAAGAAAGAT**CAG** GCAAGACAAG-3′, Mut$_{Pro111}$ 5′-GACCAAAGAAAGATAGA**C**CAAGACAAG-3′, Mut$_{Lys110–Pro111}$ 5′-GACCAAAGAAAGATA**AGC**CAAGACAAG (nucleotide differences between VEGF$_{165}$-Wt and mutants are indicated in bold) (MWG-Biotech, Ebersberg, Germany). VEGF$_{165}$ mutants were generated using the Gene Editor™ in vitro site-directed mutagenesis system (Promega, Mannheim, Germany). VEGF$_{165}$ mutants were characterized by DNA sequencing using the ABI Prism™ Big Dye™ terminator cycle sequencing ready reaction kit (PE Applied BioSystems, Langen, Germany) and confirmed in both the 5′ and the 3′ direction.

2.2. Cell culture

COS-1 cells (provided by Dr. Ingo Flamme, ZMMK, Cologne, Germany) were cultured in Dulbecco's modified-Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), L-glutamine (2 mM) and penicillin (10 U/ml) streptomycin (10 μg/ml). Human umbilical venous endothelial cells (HUVEC; TEBU, Frankfurt, Ger-

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Abbreviations: VEGF, vascular endothelial growth factor; HUVEC, human umbilical venous endothelial cells; FCS, fetal calf serum; Wt, wild type; Mut, mutant

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many) were maintained in MCDB 131 (Life Technologies, Eggenstein, Germany) supplemented with 8% FCS, 20 mM glutamine, penicillin (10 U/ml), streptomycin (10 μg/ml) and ECGS/H growth supplement (PromoCell, Freiburg, Germany).

2.3. Purification of $VEGF_{165}$-protein

COS-1 cells were transfected using the Superfect transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions and cultivated in serum free medium (DMEM containing 2 mM L-glutamine, penicillin 10 U/ml, streptomycin 10 μg/ml and ITS supplement) (Sigma, Deisenhofen, Germany). Conditioned medium (200 ml) was collected following 48 h and incubated for 4 h with 5 ml of heparin-sepharose (Pharmacia, Freiburg, Germany) at 4°C. The heparin-sepharose was packed in a column and culture medium was loaded onto the column at a flow rate of 25 ml/h. Elution of bound proteins was carried out by 10 mM Tris–HCl, 0.9 M NaCl pH 7.2. Fractions were pooled, desalted (D-Salt™ Excellulose™ Plastic Desalting Columns, Pierce, St. Augustin, Germany), lyophilized and the concentration of $VEGF_{165}$ was determined using a commercially available human VEGF-specific ELISA (R&D Systems, Minneapolis, MN, USA). The assay was performed following the manufacturer's instructions.

2.4. SDS–PAGE and immunoblotting

SDS–PAGE was performed following the protocol of Laemmli. Purchased recombinant human $VEGF_{165}$ ($rVEGF_{165}$; R&D Systems, Minneapolis, MN, USA) and $VEGF_{165}$ expressed in COS-1 cells were incubated at 37°C (pH 7.6, up to 4 h) with wound fluid obtained from non-healing wounds, plasmin solution (0.01 U/ml; human plasma plasmin, Sigma, Deisenhofen, Germany) or as a negative control with PBS. Reactions were terminated by the addition of Pefabloc® (1 mM final concentration), and frozen at −20°C before analysis by SDS–PAGE or MALDI-TOF-mass-spectrometry. For Western blotting fragments were resolved on 12% non-reducing SDS–PAGE gels and transferred to nitrocellulose (Hybond C-super, Amersham). $VEGF_{165}$ integrity was determined by detecting immunoreactive products with a polyclonal rabbit antibody that recognizes the amino-terminal VEGF epitopes (raised against a 20-amino-acid amino-terminal peptide; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection of intact and VEGF-derived degradation products was accomplished using the LumiLight plus chemiluminescence detection system (Roche Diagnostics, Mannheim, Germany). Densitometric analysis of Western blots was performed using a FluroS-Multiimager and Quantity one analysis-software (Biorad, Munich, Germany).

2.5. Protein sequencing

Protein sequencing was performed by Dr. Karlheinz Mann (Max-Planck-Institute of Biochemistry, Martinsried, Germany). For amino-terminal sequencing the PAGE-separated fragments were blotted onto Immobilon-P (Millipore, Bedford, MA, USA) according to Matsudaira [18]. Sequencing was done using the Applied BioSystems Procise 492 sequencer.

2.6. MALDI-TOF mass spectrometry

The mass spectrometry analysis of plasmin-induced VEGF degradation was performed by Dr. Macht in the Center of Molecular Medicine Cologne. For the detection of high molecular weight proteins a voltage of 20kV was used. The analysis of small molecular weight proteins was performed using a post source decay mass spectrometry (PSD-MS) with a voltage of 26.3 kV and a reflector-voltage of 22.5 kV.

2.7. Endothelial cell proliferation assay for VEGF

HUVECs were seeded in 96-well plates (4×10^3 cells/well) in growth medium. After a starvation phase of 16 h (1% FCS), cells were stimulated by VEGF variants (1 to 15 ng/ml) for 5 days. Cell proliferation was assessed using a BrdU-based proliferation assay (Roche Diagnostics). VEGF-mediated mitogenic activity was inhibited by adding a specific VEGF-neutralizing polyclonal goat antiserum (50 μg/ml) (R&D Systems). Three separate dose response experiments were performed. Differences between wild type and mutant proteins were analyzed using the unpaired *t*-test.

2.8. Wound fluid

Wound fluid was obtained from three different patients suffering from non-healing wounds due to venous insufficiency (ulcers showed no clinical sign of infection, 25–50 cm^2 in size, mean age of patients 67 years). Ulcers were covered with a semi-permeable polyurethane film (Hyalofilm®, Hartmann; Heidenheim, Germany). Following collection, fluids were centrifuged (10 min, $13\,000\times g$, 4°C) and supernatants were frozen at −80°C until use.

3. Results

Plasmin-mediated degradation of $VEGF_{165}$ was assessed using $VEGF_{165}$ protein either purified from COS-1 cell conditioned media ($VEGF_{165}$-Wt) or recombinant $VEGF_{165}$ commercially available and isolated from a baculovirus expression system ($VEGF_{165}$-Wt, R&D). As indicated by Western blotting baculovirus-expressed $VEGF_{165}$ appeared on SDS–PAGE as a single 42-kDa protein band (Fig. 1A, lanes 1–5), while $VEGF_{165}$ expressed in COS-1 cells appeared as a 42/45-kDa protein doublet (Fig. 1A, lanes 6–10). Generation of this doublet is due to protein glycosylation in the eukaryotic system. However, this posttranslational modification does not affect the protein's biological activity (Dr. Ingo Flamme, personal communication). Amino-terminal sequencing of $VEGF_{165}$ fragments obtained following plasmin digestion gave four $VEGF_{165}$ fragments. Based on the sequence-ARQENP(C)GP the most N-terminal-proximal cleavage site was identified as Arg110/Ala111 (data not shown). Other cleavage sites were between Arg123/Arg124, Lys125/His126, and Lys147/Ala148. The cleavage site Arg110/Ala111 was confirmed by MALDI-TOF analysis of digestion products (data not shown). In addition, the amino-terminal fragment VEGF1-110 could be detected as a 38-kDa fragment by Western blotting (Fig. 1A).

Oligonucleotide primer-directed mutagenesis was used to generate $VEGF_{165}$ mutants. Arg110 was changed to either Ala or Gln, and Ala111 to Pro. To test the stability of these mutants, proteins were incubated with plasmin and the degradation analyzed by Western blotting. Comparable to the COS-1-derived $VEGF_{165}$-Wt, all VEGF mutants appeared on SDS–PAGE as a 42/45 kDa protein doublet (Fig. 1B). In contrast to the susceptibility of $VEGF_{165}$-Wt to plasmin proteolysis, the electrophoretic mobility of the mutant proteins remained stable over the 240 min incubation period (Fig. 1B, lanes 1–17). $VEGF_{165}$-Wt remained stable when incubated at 37°C in buffer alone (Fig. 1B, lanes 18, 19).

Next, the mitogenic activity of each VEGF variant was assessed using a BrdU proliferation assay on HUVE cells. All mutant $VEGF_{165}$ proteins exhibited a dose-dependent stimulation of BrdU incorporation in HUVE cells and the activity of mutant proteins did not show a clear statistical difference compared to the wild type protein (Fig. 2). Mitogenic activity of wild type and mutant $VEGF_{165}$ proteins was inhibited by addition of a neutralizing polyclonal antibody to hVEGF (data not shown).

BrdU proliferation assay was used to assess the mitogenic activity of plasmin-digested $VEGF_{165}$ proteins on HUVECs. Plasmin-digestion of $VEGF_{165}$-Wt, either COS-1-derived or purchased, reduced its stimulatory effect approximately 40 and 60% following a 90 or 240-min incubation in plasmin, respectively (Fig. 3A, B). In contrast, plasmin did not affect the mitogenic potency of VEGF mutants Mut_{Ala110} and Mut_{Pro111} at any of the time points assessed (Fig. 3C,D). The mitogenic activity of VEGF-Wt protein and mutant VEGF proteins incubated at 37°C in buffer alone was stable.

To assess the stability of VEGF variants under more clin-

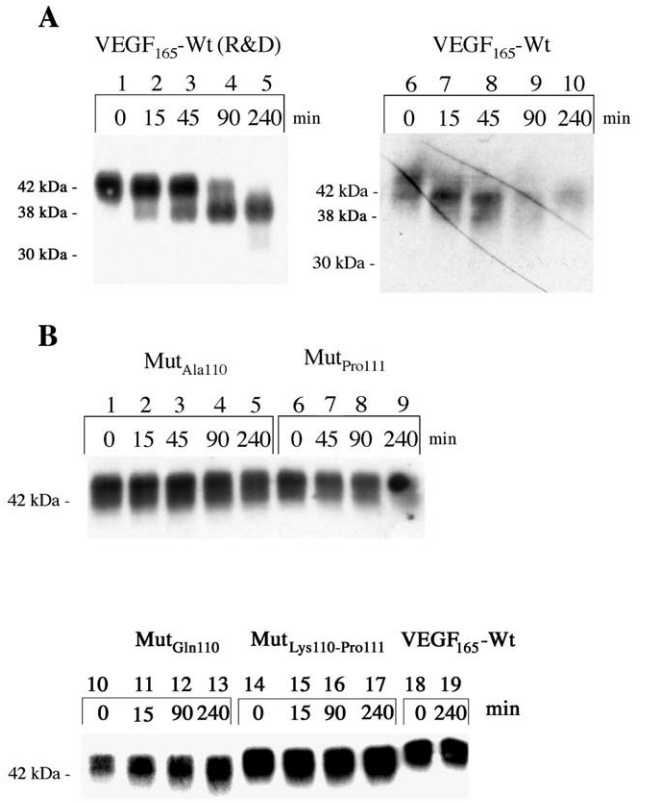

Fig. 1. Commercially available $VEGF_{165}$ ($VEGF_{165}$-Wt, R&D) (A, lanes 1–5), wild type $VEGF_{165}$ expressed in COS-1 cells ($VEGF_{165}$-Wt) (A, lanes 6–10), and $VEGF_{165}$ mutations Mut_{Ala110} (B, lanes 1–5), Mut_{Pro111} (B, lanes 6–9), Mut_{Gln110} (B, lanes 10–13) and $Mut_{Lys110–Pro111}$ (B, lanes 14–17) were incubated in plasmin for the indicated time periods. $VEGF_{165}$-Wt incubated in buffer alone (B, lanes 18 and 19) served as control. $VEGF_{165}$ degradation was monitored by SDS–PAGE under non-reducing conditions and by detecting immunoreactive products with a VEGF-specific antibody that recognizes the amino-terminal VEGF epitopes.

ically relevant conditions, wild type and mutant $VEGF_{165}$ proteins were incubated in wound fluid collected from patients suffering from non-healing wounds. In accordance with previous studies, incubation of $VEGF_{165}$ in wound fluid obtained from non-healing wounds resulted in the degradation of the 42/45 kDa proteins into a fragment of approximately 38 kDa (Fig. 4A). Interestingly, one of the mutant proteins remained stable when incubated in wound fluid obtained from non-healing wounds. The insertion of a neutral, non-cyclic amino acid at position 110 (Mut_{Ala110} and Mut_{Gln110}) leads to a degradation pattern similar to the wild type proteins – the 42/45 kDa doublet produced a single 38-kDa fragment (Fig. 4B, lanes 5–8, lanes 13–16). In contrast, substitution of alanine at position 111 with the neutral and cyclic amino acid proline produced a unique result. As indicated by Western blot, the 42/45-kDa doublet remained stable in the presence of wound fluid over the 1-h incubation period (Fig. 4B, lanes 1–3, lanes 9–11). Prolonged incubation of Mut_{Pro111}/$Mut_{Lys110-Pro111}$ up to 240 min led to a protein smear over 45 and 38 kDa (Fig. 4B, lanes 4, 12). The stability of Mut_{Pro111} and the degradation pattern of Mut_{Ala110} and Mut_{Gln110} proteins were consistent in wound fluids obtained from three different patients. Western blots of $VEGF_{165}$-Wt protein and $Mut_{Lys110-Pro111}$ protein digested in wound fluid were analyzed by scanning densitometry (Fig. 4C).

4. Discussion

In the present study, we elucidated the functional role of the plasmin cleavage site Arg110/Ala111 in the $VEGF_{165}$ molecule for its mitogenic potency. Western blot analysis, amino-terminal sequencing and MALDI-TOF analysis of plasmin-digested $VEGF_{165}$ fragments identified Arg110/Ala111 as the plasmin cleavage site closest to the N-terminus of the molecule. Incubation of $VEGF_{165}$ in plasmin for 4 h reduced its mitogenic potency on HUVE cells by over 50%. This data suggests a

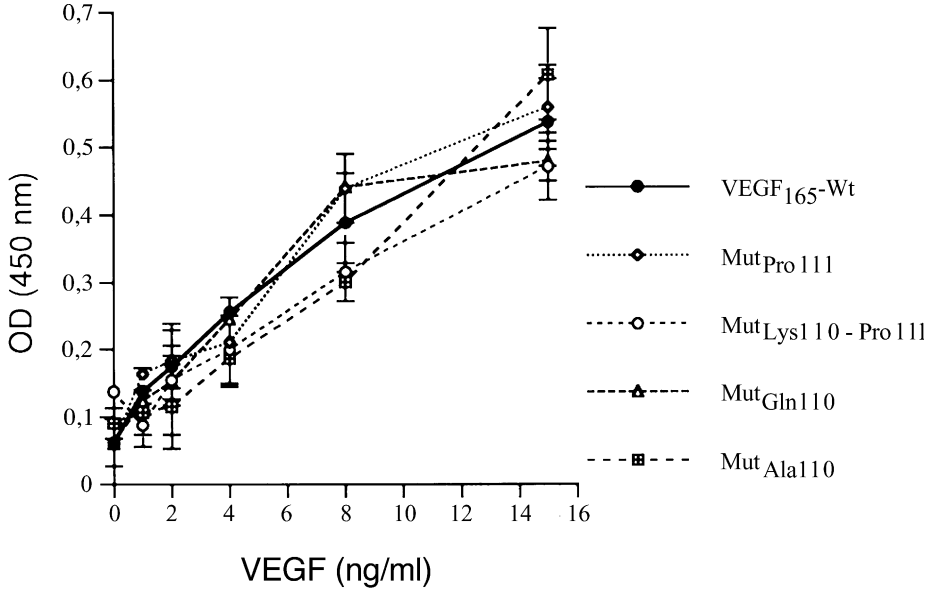

Fig. 2. HUVECs were cultured for 96 h in the presence of varying concentrations of $VEGF_{165}$ variants expressed in COS-1 cells. BrdU incorporation in proliferating cells was determined by ELISA. Triplicate samples were assayed and results are shown ± S.D.

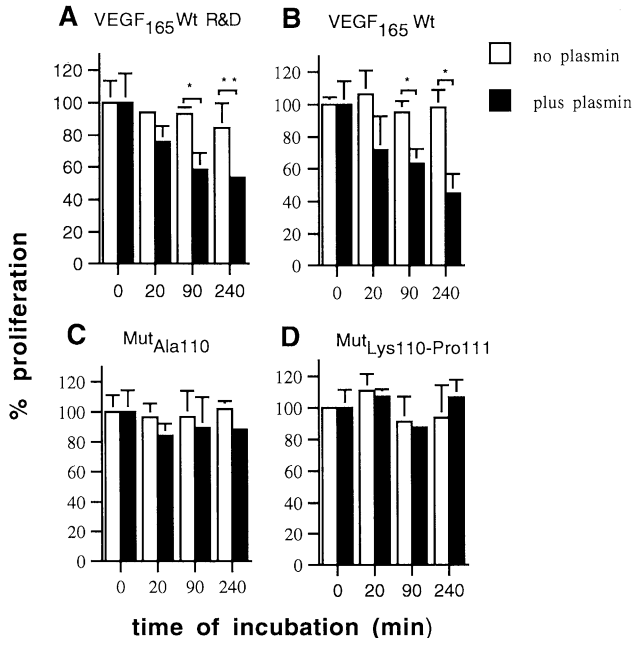

Fig. 3. Commercially available $VEGF_{165}$ ($VEGF_{165}$-Wt, R&D) (A), $VEGF_{165}$-Wt expressed in COS-1 cells ($VEGF_{165}$-Wt) (B), and mutant $VEGF_{165}$ proteins Mut_{Ala110} (C) and $Mut_{Lys110–Pro111}$ (D) were incubated in plasmin for the indicated time ($VEGF_{165}$ 10 ng/ml). Following plasmin incubation, the mitogenic potency of $VEGF_{165}$ proteins was determined on HUVECs. BrdU incorporation in proliferating cells was determined by ELISA. Statistical analysis was performed with the unpaired *t*-test. Triplicate samples were assayed and results are shown ± S.D. (*$P = 0.0275$, **$P \leq 0.0084$).

critical role of the plasmin cleavage site Arg110/Ala111 and the carboxyl-terminal domain of $VEGF_{165}$ in the stimulation of endothelial cell proliferation.

VEGF activity is mediated through two receptors: flt-1 and flk-1/KDR [4]. Both receptors show different kinase activity, and have separate signal transduction properties and possibly mediate different functions [4,19,20]. Receptor studies suggest that the major positive signal of VEGF for endothelial proliferation and vascular permeability appears through flk-1/KDR, while that from flt-1 seems to contribute about one tenth of the total signal [21,22]. Recently, Soker et al. have identified a new VEGF receptor, neuropilin-1, that binds $VEGF_{165}$ but not $VEGF_{121}$ [7]. When co-expressed in cells with flk-1/KDR, neuropilin-1 enhanced the binding of $VEGF_{165}$ to flk-1/KDR and $VEGF_{165}$-mediated chemotaxis and proliferation [7,23]. Conversely, inhibition of $VEGF_{165}$ binding to neuropilin-1 inhibits its binding to flk-1/KDR and its mitogenic activity for endothelial cells. These findings suggest that neuropilin-1 may present $VEGF_{165}$ to the flk-1/KDR receptor in a manner that enhances the effectiveness of flk-1/KDR-mediated signal transduction. $VEGF_{165}$ binds to neuropilin-1 via the VEGF exon 7 encoded peptide, which comprises most of the heparin-binding site of $VEGF_{165}$. The

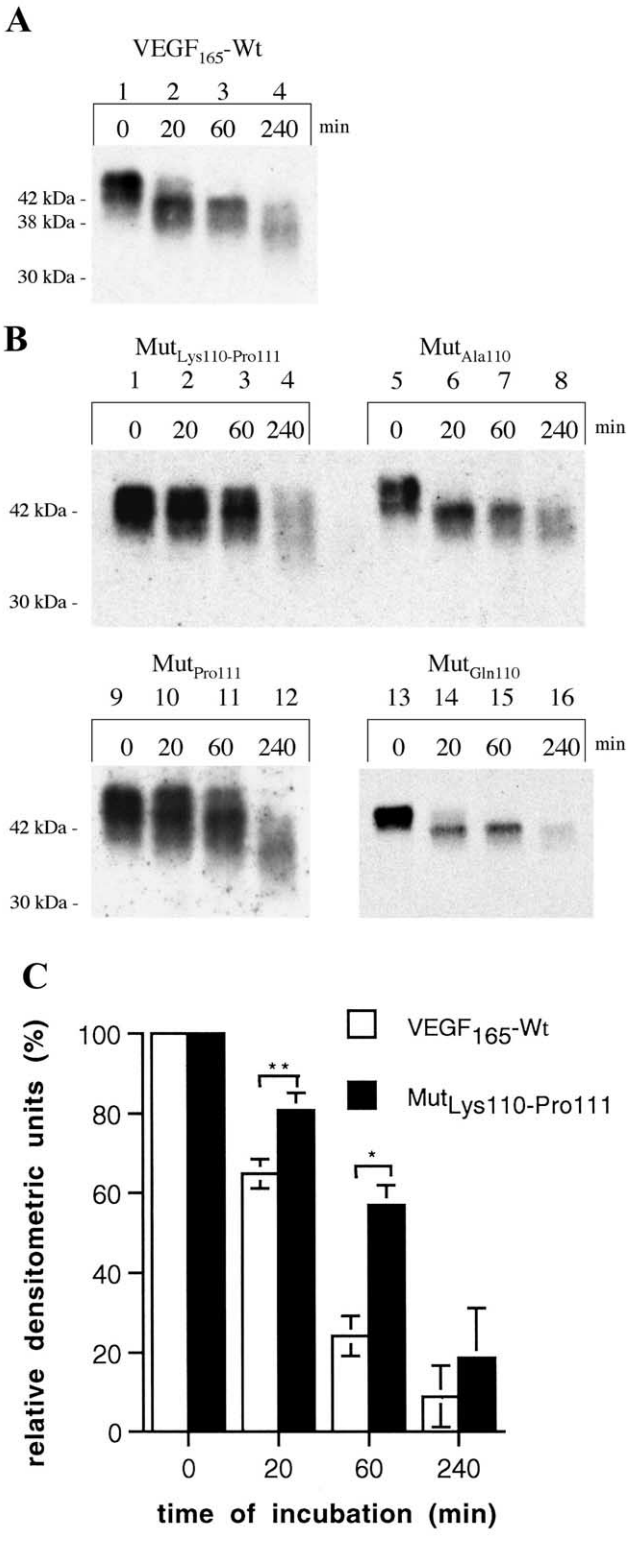

Fig. 4. $VEGF_{165}$-Wt (A) and mutant $VEGF_{165}$ proteins $Mut_{Lys110–Pro111}$ (B, lanes 1–4), Mut_{Ala110} (B, lanes 5–8), Mut_{Pro111} (B, lanes 9–12) and Mut_{Gln110} (B, lanes 13–16) were incubated in wound fluid collected from patients suffering from chronic non-healing wounds for time periods as indicated (patients $n = 3$; representative Western blots are presented). $VEGF_{165}$ degradation was monitored by SDS–PAGE under non-reducing conditions and by detecting immunoreactive products with a VEGF-specific antibody that recognizes the amino-terminal VEGF epitopes. C: Degradation of $VEGF_{165}$-Wt and $Mut_{Lys110–Pro111}$ protein incubated in chronic wound fluid was monitored by analyzing the intensity of the 42/45 kDa signal by scanning densitometry, and is presented as a percentage of the signal intensity at timepoint 0. Statistical analysis was performed with the unpaired *t*-test. Three Western blots were analyzed and results are shown ± S.D. (*$P = 0.0055$, **$P = 0.0082$).

ability of $VEGF_{165}$ to bind neuropilin-1 may partly explain its greater mitogenic potency relative to VEGF molecules missing the exon 7 encoded peptides, whether due to proteolysis or alternative splicing.

Recently we demonstrated that proteolysis and inactivation of $VEGF_{165}$ by plasmin is increased in wound fluid collected from non-healing wounds versus healing wounds [14]. These results prompted us to investigate whether $VEGF_{165}$ could be proteolytic stabilized by mutating the plasmin cleavage site Arg110/Ala111. A general consensus sequence of the plasmin cleavage site is not known. Alterations in amino acid residues of $VEGF_{165}$ were based on the notion that plasmin substrate specificity is directed by its negatively charged catalytic domain. Therefore, Arg110 was substituted by a neutral, non-polar, non-cyclic amino acid such as alanine or glutamine. We also replaced Ala111 with proline, an amino acid change that might be expected to have effects on the tertiary as well as primary structure of the protein. Western blot analysis and cell proliferation assay demonstrated that both the replacement of the Arg110 by a neutral, non-cyclic amino acid and the introduction of Pro111 stabilized $VEGF_{165}$ mitogenic activity in the presence of plasmin. This data indicates that the mutation of the Arg110/Ala111 site of $VEGF_{165}$ dramatically affected its plasmin sensitivity.

Consistent with these observations, $VEGF_{165}$ mutants were temporarily stabilized when incubated with wound fluid obtained from non-healing wounds. However, inactivation of the plasmin cleavage site by a neutral, non-cyclic amino acid is not sufficient to stabilize the $VEGF_{165}$ molecule in the presence of wound fluid. In contrast, altering the plasmin cleavage site by substituting VEGF-Ala111 with the cyclic VEGF-Pro111 increases the VEGF stability in chronic wounds. These results suggest that modification of the tertiary structure of the $VEGF_{165}$ protein can temporarily stabilize $VEGF_{165}$ in the wound fluid of non-healing wounds. However, a direct effect at the level of primary sequence recognition by proteases is also possible.

Little is known about local mechanisms leading to and perpetuating cutaneous wound healing failure. A disturbed balance of proteolytic and anti-proteolytic activity characterizes the hostile environment of a chronic wound [24,25]. The failure of certain wounds to resolve successfully may be due to the increased proteolytic degradation of regulatory factors, including growth factors and extracellular matrix molecules [14,26–28]. Increased degradation of topically applied growth factors may also account for the discrepancy between the remarkable efficacy of growth factors in wound healing in animal models and the rather disappointing clinical results in non-healing wounds [14,27]. Hence, controlling the proteolytic activity in the chronic wound environment might be a key strategy to improve wound healing. Topical application of proteolysis-resistant growth regulators in chronic wounds might enhance the efficiency of these mediators in the hostile chronic wound environment.

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References

[1] Brown, L.F., Detmar, M., Claffey, K., Nagy, J.A., Feng D., Dvorak, A.M., Dvorak, H.F. (1997) in: Regulation of Angiogenesis (Goldberg, I.D., Rosen, E.M., Eds.), pp. 233–269, Birkhäuser, Basel.
[2] Tischer, E., Mitchell, R., Hartmann, T., Silvia, M., Gospodarowicz, D., Fiddes, J.C. and Abraham, J.A. (1991) J. Biol. Chem. 266, 11947–11954.
[3] Lei, J., Jiang, A. and Pei, D. (1990) Biochem. Biophys. Acta 1443, 400–406.
[4] Shibuya, M. (2001) Cell Struct. Funct. 26, 25–35.
[5] Park, J.E., Keller, G.-A. and Ferrara, N. (1993) Mol. Biol. Cell 4, 1317–1326.
[6] Tessler, S., Rockwell, P., Hicklin, D., Cohen, T., Levi, B., Witte, L., Lemischka, I.R. and Neufeld, G. (1994) J. Biol. Chem. 269, 12456–12461.
[7] Soker, S., Takashima, S., Miao, H.Q., Neufeld, G. and Klagsbrun, M. (1998) Cell 92, 735–745.
[8] Gitay-Goren, H., Soker, S., Vlodavsky, I. and Neufeld, G. (1992) J. Biol. Chem. 267, 6093–6098.
[9] Gitay-Goren, H., Cohen, T., Tessler, S., Soker, S., Gengrinovich, S., Rockwell, P., Klagsbrun, M., Levi, B.Z. and Neufeld, G. (1996) J. Biol. Chem. 271, 5519–5523.
[10] Houck, K.A., Leung, D.W., Rowland, A.M., Winer, J. and Ferrara, N. (1992) J. Biol. Chem. 267, 26031–26037.
[11] Ortega, N., L'Faqihi, F.-E. and Plouet, J. (1998) Cell 90, 381–390.
[12] Plouet, J., Moro, F., Bertagnolli, S., Coldeboeuf, N., Mazarguil, H., Clamens, S. and Bayard, F. (1997) J. Biol. Chem. 272, 13390–13396.
[13] Keyt, B.A., Berleau, L.T., Nguyen, H.V., Chen, H., Heinsoh, H., Vandlen, R. and Ferrara, N. (1996) J. Biol. Chem. 271, 7788–7795.
[14] Lauer, G., Sollberg, S., Cole, M., Flamme, I., Stürzebecher, J., Mann, K., Krieg, T. and Eming, S.A. (2000) J. Invest. Dermatol. 115, 12–18.
[15] Brown, L.F., Yeo, K.T., Berse, B., Senger, D.R., Dvorak, H.F. and Van De Water, L. (1992) J. Exp. Med. 176, 1375–1379.
[16] Howdieshell, T.R., Callaway, D., Webb, W., Gaines, M.D., Procter, C.D., Sathyanarayana, Pollock, J.S., Brock, T.L. and McNeil, P.L. (2001) J. Surg. Res. 96, 173–182.
[17] Kishimoto, J., Ehama, R., Ge, Y., Kobayashi, T., Nishiyama, T., Detmar, M. and Burgeson, R.E. (2000) Am. J. Path. 157, 103–110.
[18] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[19] Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.H. (1994) J. Biol. Chem. 269, 26988–26995.
[20] Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S. and Shibuya, M. (1995) Oncogene 10, 135–147.
[21] Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y. and Shibuya, M. (1998) J. Biol. Chem. 273, 31273–31282.
[22] Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H.G., Ziche, M., Lanz, C., Buttner, M., Rziha, H.J. and Dehio, C. (1999) EMBO J. 18, 363–374.
[23] Miao, H.-Q. and Klagsbrun, M. (2000) Cancer Metastasis Rev. 19, 29–37.
[24] Falanga, V., Grinnell, F., Gilchrest, B., Maddox, Y. and Moshell, A. (1994) J. Invest. Dermatol. 102, 125–127.
[25] Singer, A.J. and Clark, R.A. (1999) New Engl. J. Med. 341, 738–746.
[26] Grinnell, F., Ho, C.H. and Wysocki, A. (1992) J. Invest. Dermatol. 98, 410–416.
[27] Wlaschek, M., Peus, D., Achterberg, V., Meyer-Ingold, W. and Scharffetter-Kochanek, K. (1997) Br. J. Dermatol. 137, 646.
[28] Barrick, B., Campbell, E.J. and Owen, C.A. (2000) Wound Repair Regen. 7, 410–422.