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Novel Glycosylated Forms of Human Plasma Endostatin and Circulating Endostatin-Related Fragments of Collagen XV†

Harald John,‡ Klaus T. Preissner,§ Wolf-Georg Forssmann,‡ and Ludger Ständker*,‡

Lower Saxony Institute for Peptide Research (IPF), Feodor-Lynen-Strasse 31, D-30625 Hannover, Germany, and Institute for Biochemistry, Medical Faculty, Justus-Liebig-University, D-35392 Giessen, Germany

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ABSTRACT: Circulating elongated forms of the angiogenesis inhibitor and potential anti-cancer drug endostatin were isolated from human blood filtrate. Immunoreactive endostatin was identified by a polyclonal rabbit antiserum raised against an N-terminal epitope of the polypeptide and purified by consecutive chromatographic steps and immunoblotting. N- and C-terminal sequence analyses of the isolated molecules revealed different forms of endostatin starting with V^{117}HLRPAR... lacking the last and final three residues of the noncollagenous domain 1 (NC-1) of collagen XVIII, respectively. These polypetides are found to be O-glycosylated at T^{125} (residue 9) with a glycan structure of the mucin type consisting of galactose *N*-acetylgalactosamine and *N*-acetylneuraminic acid residues. Carbohydrate analyses were performed via the semiquantitative HPLC−electrospray ionization mass spectrometry (ESMS) technique after exoglycosidase hydrolysis. Circulating endostatins are present as sialoglycoprotein (22 000 and 21 841 Da ± 0.02%) and asialoglycoprotein structures (21 710 and 21 549 Da ± 0.02%), while the two completely deglycosylated forms are obtained only after enzymatic incubation. The described glycosylated endostatins may represent intermediates in the proteolytic pathway of the NC-1 domain of collagen XVIII resulting in bioactive endostatins. Furthermore, immunoreactive endostatin-related C-terminal fragments of human collagen XV are found in the hemofiltrate. These polypeptides exhibit the N-terminal sequences P^{66}HLLPPP... and Y^{81}EKPALH... of the collagen XV NC-1 domain. ESMS and immunoblotting analyses reveal three glycosylated polypeptides with a molecular mass ranging from 16 to 21 kDa. Due to the high degree of homology between collagen XV and collagen XVIII as well as their analoqous proteolytic processing, functional similarities of collagen XVIII- and XV-related fragments should be revealed in future experiments.

Processes of angiogenesis, the sprouting of new capillaries from preexisting blood vessels, are observed not only at sites of tissue or vascular injury, during the menstrus cycle, or upon pregnancy (*1*) but also in pathological situations such as during collateral formation in ischemic (heart) tissue, with skin diseases, during the development of diabetic retinopathy, or during the progression of solid tumors (*2*). Tumor angiogenesis also enables the release of daughter cells from the primary tumor site and thereby favors metastasis (*3*). The extent of angiogenesis particularly mediated through the

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* To whom correspondence should be addressed. Phone: +49-(0)-511-5466-329. Fax: +49-(0)511-5466-132. E-mail: L.standker@gmx.net.

‡ Lower Saxony Institute for Peptide Research (IPF).

§ Justus-Liebig-University.

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degree of proliferation, migration, and lumen formation of endothelial cells is dependent on recently defined cell−matrix interactions that control the survival or apoptosis of endothelial cells in an integrin-related fashion (*4*−*7*). These processes can be correlated with the spatiotemporal expression or availability of various pro- and antiangiogenic factors, some of which have been identified as proteolytic fragments of existing larger precursor proteins in the body. Recent examples of potent intrinsic antiangiogenic factors include angiostatin (kringle-fragments of plasminogen) (*8*), thrombospondin fragments (*9*), and fragments of matrix metalloproteinases (*10*) as well as endostatin(s) (*11*) that represent C-terminal fragments of the NC-1 domain of collagen XVIII (*12*), expressed in vessels and other tissues (*13*), or in the liver (*14*). Angiostatin and endostatin appear to specifically block (tumor) angiogenesis in different animal models without obvious side effects (*8*, *15*−*17*), yet the mechanisms of their proteolytic release and their mode of action remain largely undefined.

Recombinant endostatin material has been produced from several sources, including human embryonic kidney cells (*18*), *Escherichia coli*, baculovirus expression systems (*16*), or yeast *Pichia pastoris* (*19*, *20*). Both recombinant mouse and human endostatin inhibited the growth of different primary tumors in mouse such as Lewis lung carcinoma, T241 fibrosarcoma, hemangioendothelioma (EOMA),[1] or B16F10 melanoma. Furthermore, the regression of tumors to a microscopic size and their lingering in a dormant state after systemic administration of endostatin combined with the lack of acquired drug resistance could make this polypeptide a potential anti-cancer drug (*17*). Due to the high degree of identity of 85% between murine and human endostatin and their high degree of homology of 99% (*21*), their functional potency might be similar.

Screening a peptide bank established from 10 000 L of human blood filtrate obtained from patients with renal disease (*22*, *23*), we were able to isolate a circulating form of endostatin as a 18.5 kDa molecule that lacks the first 12 amino acids compared to the mouse sequence (*11*, *24*). Moreover, additional forms of endostatin have been isolated from mice and men differing in their size and in their N-terminal sequences. In the work presented here, using a combination of immunoblotting and consecutive reversed-phase (RP) chromatographies, we identified and purified six elongated endostatin polypeptides (21.2−22 kDa) differing in their glycosylation and C-terminal extensions. In addition, endostatin-related C-terminal fragments of human collagen XV were discovered during chromatographic purification of immunoreactive endostatin polypeptides from blood filtrate. These fragments, dubbed restin by Ramchandran et al. (*25*), were shown to possess antiangiogenic activity in vitro and in vivo when expressed as recombinant material. These data emphasize the fact that limited proteolytic processing of host proteins such as collagens XVIII and XV results in the generation of bioactive fragments that participate in the control of physiological and pathological angiogenesis. The generation of various polypeptide forms in each case may reflect the sensitivity toward as yet unidentified protease(s) whose profile may change with different pathological situations.

[1] Abbreviations: CZE, capillary zone electrophoresis; col-XV, collagen α1 (XV); col-XVIII, collagen α1 (XVIII); EOMA, hemangioendothelioma; ESMS, electrospray ionization mass spectrometry; Gal, galactose; GlcNAc, *N*-acetylglucosamine; HF, hemofiltrate; NAc, *N*-acetyl; NANA, *N*-acetylneuraminic acid; NC, noncollagenous; RP, reversed-phase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Chromatographic Isolation of Endostatin Proteins. Ten thousand liters of human blood ultrafiltrate (hemofiltrate, HF) from patients with chronic renal insufficiency was extracted and prepared to generate a peptide bank according to Schulz-Knappe et al. (*22*, *23*) using cation exchange and RP chromatography (steps 1 and 2). Lyophilized aliquots of these fractions corresponding to 2 L of HF each were used to detect endostatin proteins by a reducing Western blot analysis. Fractions with intensive immunoreactivity corresponding to an apparent molecular mass of 26 kDa were pooled and subjected to further RP chromatography on a C18 Prepak cartridge (300 mm × 47 mm inside diameter, 300 Å, 15−20 μm, Vydac, Waters, Milford, MA). All RP chromatographic separations were carried out at room temperature and monitored at 214 and 280 nm. Elution of proteins was performed using a rising methanol gradient at a rate of 40 mL/min as indicated in Figure 1B. The mobile phase was comprised of eluent A_3 (20:80 v/v MeOH/H_2O and 0.01 M HCl) and eluent B_3 (0.01 M HCl in MeOH) (step 3). Fractions containing the most intensive endostatin immunoreactivity were pooled and rechromatographed using an analytical RP C4 column (250 mm × 10 mm inside diameter, 300 Å, 5 μm, Vydac, Hesperia, CA). The chromatography was performed with a linear gradient using 0.1% TFA in H_2O as eluent A_4 and a mixture of CH_3CN, H_2O, and TFA (80:20:0.1 v/v/v) as eluent B_4 as shown in Figure 1C (step 4). At this stage of purification, immunoreactive endostatin proteins were detected by ESMS with an apparent molecular mass in the range of 22 kDa. The last step of purification was performed on an analytical RP C18 column (250 mm × 10 mm inside diameter, 120 Å, 5 μm, YMC, Schermbeck, Germany) using eluents A_4 and B_4 (flow rate of 1.5 mL/min, gradient from 30 to 80% B_4 over the course of 63 min, fraction size of 1.5 mL) (step 5). The 22 kDa immunoreactive proteins were found in three fractions (fractions 27−29), as detected by UV absorption and ESMS. The purity was investigated by capillary zone electrophoresis (CZE) and N-terminal sequencing.

Generation of Antibodies and Western Immunoblotting. For antisera generation, female white New Zealand rabbits were immunized (approved by a local ethics commitee 96/908) using an octameric multiple antigenic peptide (MAP) (*26*) containing the 15 N-terminal residues of the human 18.5 kDa endostatin sequence (V^{142}ALNSPLSGGMRGIR). For detection of immunoreactive proteins, lyophilized aliquots of the HPLC fractions were reconstituted in sample buffer [0.2 M Tris, 4% w/v sodium dodecyl sulfate, 0.02% w/v EDTA, 2% w/v dithiothreitol (DTT), 50% w/v glycerol, and 0.1% w/v bromophenol blue (pH 8.4)] and subjected to SDS−PAGE (*27*, *28*) in Mini-protean systems (Bio-Rad, Munich, Germany) at 60 V for 20 min following 150 V for 100 min. Molecular mass standards of 4−210 kDa (Seeblue; NOVEX, San Diego, CA) and recombinant human endostatin (BioVisioN, Hannover, Germany) served as references. Rabbit anti-endostatin antiserum (lot no. K304T7) was used

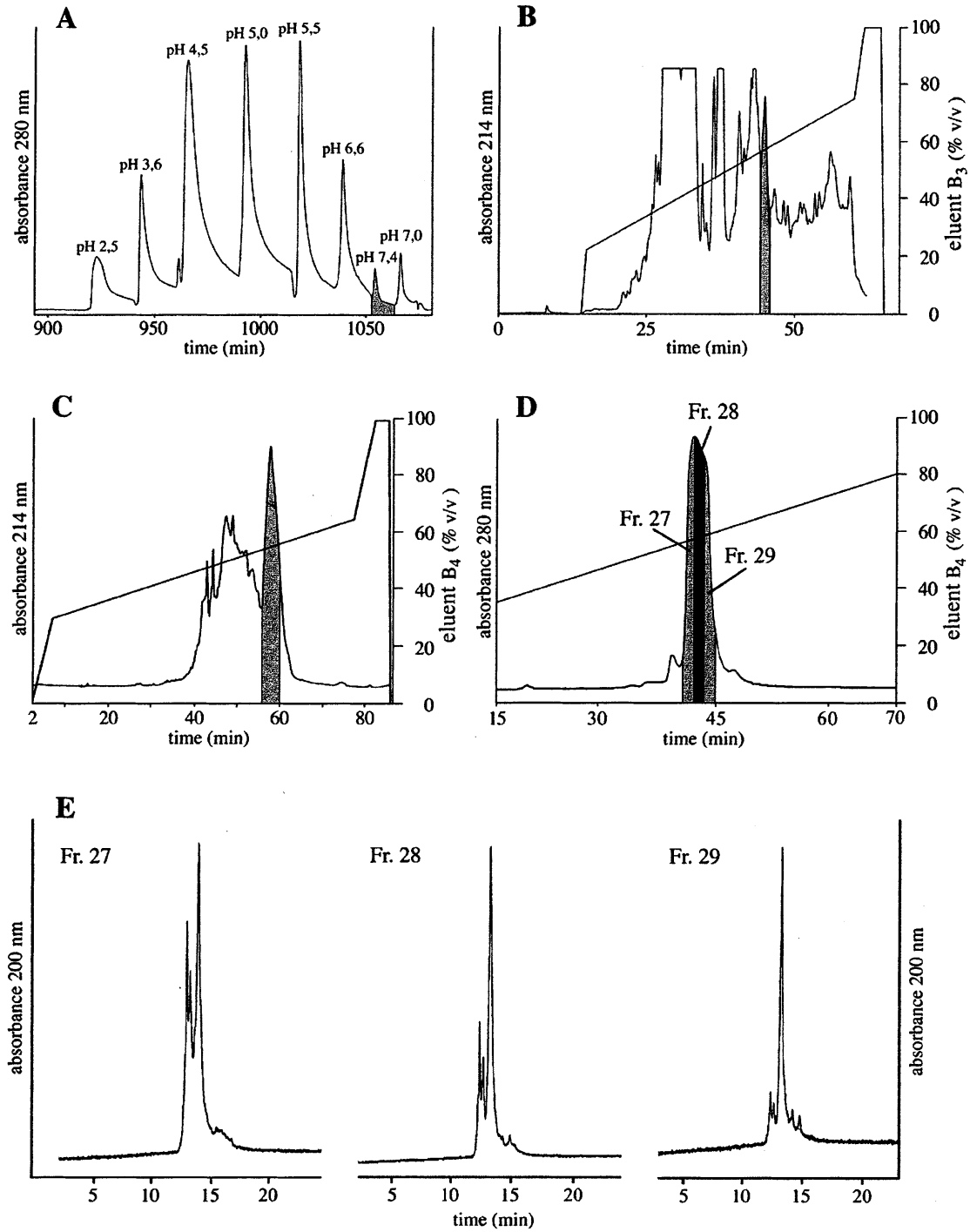


FIGURE 1: Purification of glycosylated plasma forms of endostatin. (A) Stepwise cation exchange chromatography of 10 000 L of human hemofiltrate using aqueous buffers with increasing pH values generating eight pH-pool fractions (10 L column, Vantage, TSK SP 650 M) (step 1). Each pH pool was fractionated by RP-HPLC (step 2, data not shown) to generate the peptide bank (*22*, *24*). (B) RP-HPLC fractionation of a peptide-bank fraction originating from pH pool 7 (C18 Prepak cartridge, 300 mm × 47 mm inside diameter, 300 Å, 15−20 μm; eluent A_3 being 20:80 v/v MeOH/H_2O and 0.01 M HCl and eluent B_3 being 0.01 M HCl in MeOH; gradient as indicated; rate of 40 mL/min). Fractions with endostatin immunoreactive proteins are denoted with shading (step 3). (C) RP-HPLC of the immunoreactive fractions shown in panel B (RP C4, 250 mm × 10 mm inside diameter, 300 Å, 5 μm; eluent A_4 being 0.1% TFA in H_2O and eluent B_4 being 80:20:0.1 v/v/v H_3CCN/H_2O/TFA; gradient as indicated; rate of 2 mL/min). Fractions with masses of about 22 kDa as determined by ESMS are denoted with shaded boxes (step 4). (D) Final RP-HPLC of the marked fractions shown in panel C (RP C18, 250 mm × 10 mm inside diameter, 120 Å, 5 μm; eluents A_4 and B_4 as described above; gradient as indicated; rate of 1.5 mL/min) (step 5). (E) Capillary zone electrophoresis of fractions 27−29 from panel D [fused silica uncoated capillary, 50 cm × 75 μm; 0.1 M Na_3PO_4 with 0.02% w/v (hydroxypropyl)methylcellulose; pH 2.5 and 25 °C with an *I* of 80 μA]. CZE revealed isolation of different endostatin forms each starting with V^{117}HLRPAR of the collagen XVIII NC-1 domain as confirmed by sequence analyses.

in a 1:500 dilution. Bands were developed using a bromochloroindolyl phosphate/nitroblue tetrazolium substrate system as recommended by the manufacturer (Sigma, Deisenhofen, Germany) after blot incubation with alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma).

Protein Analysis. Molecular masses of proteins were determined by ESMS using a Sciex API III quadrupole mass spectrometer (Sciex, Perkin-Elmer, Langen, Germany) with

an electrospray ionization supported by the MacSpec 3.3 software from Perkin-Elmer. Flow injection was carried out at a rate of 5 μL/min. The purity of isolated endostatin proteins was investigated by capillary zone electrophoresis (CZE) using a P/ACE system 2000 (Beckman, San Ramon, CA) supported by the accompanying System Gold software. A fused silica uncoated capillary column (50 cm $\times$ 75 μm, Polymicro Technologies, Phoenix, AZ) was used in combination with a 0.1 M sodium phosphate buffer [0.02% w/v (hydroxypropyl)methylcellulose] at pH 2.5 and 25 °C. The separation was carried out with a constant current of 80 μA, and substances were detected at 200 nm using an integrated UV detector.

N-Terminal sequencing was performed on a 473A gas phase sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin amino acids using the standard protocol recommended by the manufacturer. C-Terminal sequencing was carried out at 25 °C using sequencing grade carboxypeptidase P [0.5 μg dissolved in 200 μL of 0.1 M pyridinium acetate (pH 5.5)] purchased from Boehringer Mannheim. The consecutive standard amino acid composition analyses were performed using Amino Quant II 1090 system (Hewlett-Packard, Waldbronn, Germany) after derivatization with *o*-phthaldehyde and 9-fluorenylmethylchloroformate (Fmoc).

Carbohydrate Analysis. For the initial carbohydrate analysis, aliquots of the purified endostatin and restin proteins were analyzed with the DIG glycan differentiation kit (Boehringer Mannheim, Mannheim, Germany). Using different lectin conjugates, the differentiation of several carbohydrates was performed in a BIO-DOT apparatus (Bio-Rad) after immobilization of the proteins on a nitrocellulose membrane.

For a glycan analysis, the endostatin proteins were characterized before and after treatment with exoglycosidases by HPLC−ESMS. The proteins were redissolved in 0.02 M sodium phosphate buffer (pH 7.7, 500 μg/mL), with subsequent addition of 1 μL of buffered solutions of BSA-free *O*-glycosidase (1 munit/μL, *O*-glycopeptide endo-D-galactosyl-*N*-acetyl-β-galactosamino hydrolase, EC 3.2.1.97) or neuraminidase (1 munit/μL, acylneuraminyl hydrolase, EC 3.2.1.18) (Boehringer) in separate vials. After overnight incubation at 37 °C, the products were analyzed by HPLC−ESMS. The HPLC equipment consisted of a 140B solvent delivery system combined with a 785A programmable absorbance detector (Applied Biosystems). After separation on a Jupiter C18 column (150 mm $\times$ 1 mm inside diameter, 5 μm, 300 Å, Phenomenex, Aschaffenburg, Germany) using a linear acetonitrile gradient (0 to 80% v/v eluent B_4 over the course of 80 min, flow rate of 20 μL/min), the eluates were monitored on-line by UV absorption at 215 nm and by ESMS.

Mathematical Processing of ESMS Data. For qualitative and semiquantitative interpretation of the changed sample compositions due to enzymatic incubation of fraction 28, the ESMS results were treated as follows. Intensities of the *m*/*z* values belonging to several masses (M_1−M_4) were arranged by their charges (10−19-fold positively charged). Then the ratios of intensities belonging to the different masses for each charge were calculated, creating the relations M_1/M_2, M_1/M_3, and M_2/M_4. Via calculation of the means and standard deviations (SD) over all charges for each set of these ratios, averaged quotients were obtained, giving characteristic features for the sample compositions.

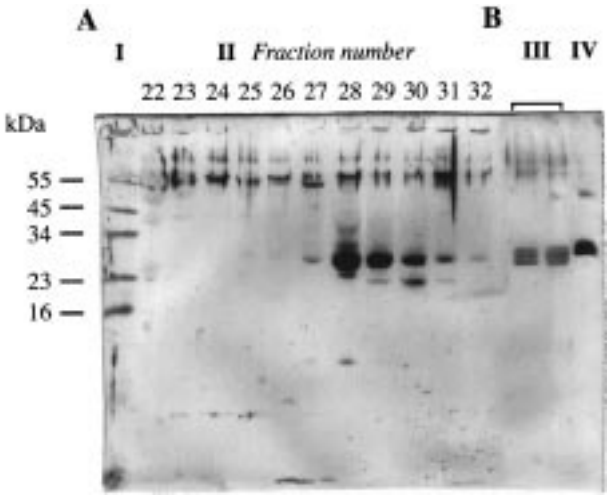
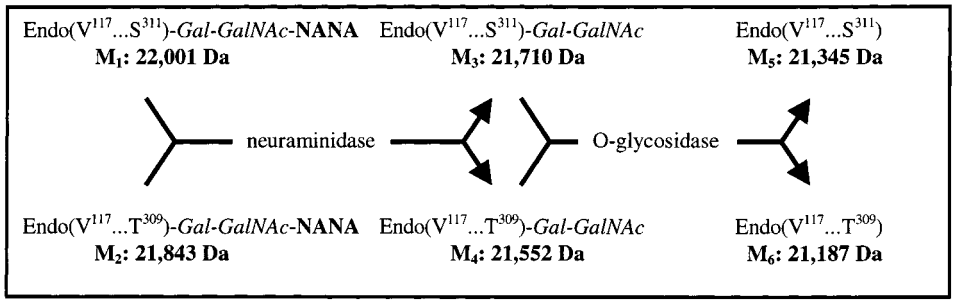


FIGURE 2: Detection of endostatin immunoreactive proteins by Western immunoblot. (A) Protein standard (I) and aliquots of the RP-HPLC fractions shown in Figure 1B (II) according to purification step 3 indicated main immunoreactive endostatin proteins in the range of 26 kDa and bands with minor intensities in the range of 18.5, 30, and 34 kDa. (B) Aliquots of a similar RP-HPLC fractionation (III) corresponding to purification step 5 (Figure 1D) revealed the existence of C-terminal collagen XV fragments in the range of 22−26 kDa. In comparison to the endostatins, these proteins eluted earlier on the reversed-phase column used for purification step 3 (Figure 1B). Recombinant endostatin (IV).

RESULTS

Purification and Analysis of Immunoreactive Endostatin Polypeptides. Extraction of peptides from 10 000 L of human hemofiltrate by cation exchange chromatography (step 1, Figure 1A) resulted in the generation of pH-pool fractions, which enabled us to establish a peptide bank of circulating peptides by RP-HPLC (step 2, results not shown) (*22*, *24*). Screening of these peptide bank fractions and of subsequently generated RP-HPLC fractions (step 3 in Figure 1B and Figure 2A) by Western immunoblotting indicated immunoreactive endostatin proteins in few fractions. Consecutive RP chromatographies of these immunoreactive fractions resulted in the purification of major immunoreactive proteins (steps 4 and 5, Figure 1C,D). CZE (Figure 1E), ESMS, and sequence analyses of the purified fractions of step 5 (fractions 27−29) indicated the occurrence of different forms of endostatin. Sequence analysis revealed VHLRPARP_SPPAHSHRDFQ (residues 117−136 of the collagen XVIII NC-1 domain) to be the N-terminal amino acids of all of the three main compounds shown in Figure 1E. Numbering of the amino acid sequence follows that of the NC-1 domain published by Sasaki et al. (*11*). The residue in sequence position 9 could not be classified. C-Terminal sequence analysis of the purest fraction (fraction 29) resulted in the sequence $FMTAS^{311}$, representing the C-terminal residues of the previously identified circulating endostatin. The purified endostatin polypeptides correspond to the broad immunoreactive band with an apparent mass of 26 kDa (Figure 2A). Further immunoreactivities were detected by Western blot analysis with apparent molecular masses of 18.5, 30, and 34 kDa, respectively. Analysis of fraction 29 by ESMS and HPLC−ESMS (mass accuracy of 0.02%) classified this major

Table 1: Carbohydrate Analysis and the Reaction Path of Purified Endostatin Peptides Containing Fraction 28 As Determined by the HPLC−ESMS Technique Combined with Enzymatic Treatment[a]



charge	M_1 (Da)	M_2 (Da)	M_3 (Da)	M_4 (Da)	M_1/M_2	M_1/M_3	M_2/M_4
10	14413	3067			4.70		
11	15453	3707	3120		4.17	4.95	
12	24560	5240	5840	1493	4.69	4.21	3.51
13	34280	7160	9133	2373	4.79	3.75	3.02
14	35747	7493	10520	2680	4.77	3.40	2.80
15	29333	5493	9627	2480	5.34	3.05	2.21
16	20933	3973	7040	1853	5.27	2.97	2.14
17	13787		5173	1387		2.67	
18	8800		3667	880		2.40	
19	5333		2453			2.17	
before enzymatic hydrolysis				mean ± SD	4.8 ± 0.4 (n = 7)	3.3 ± 0.9 (n = 9)	2.7 ± 0.6 (n = 5)
after enzymatic hydrolysis with				neuraminidase	4.7 ± 0.5 (n = 5)	1.1 ± 0.3 (n = 10)	1.2 ± 0.2 (n = 5)
				O-glycosidase	5.4 ± 0.7 (n = 6)	10.1 ± 2.9 (n = 7)	4.5 ± 1.0 (n = 5)

[a] When fraction 28 was incubated with neuraminidase, the sialoglycoproteins M_1 and M_2 were converted exclusively into the asialoglycoproteins M_3 and M_4 in the same manner (constant relation of M_1/M_2 and decreasing ratios of M_1/M_3 and M_2/M_4). In contrast, incubation with *O*-glycosidase reduced the amounts of M_3 and M_4 which are converted into M_5 and M_6 (constant ratio of M_1/M_2 and increasing ratios of M_1/M_3 and M_2/M_4). M_6 was found after incubation of fraction 27 with both enzymes mentioned above. Masses listed in the scheme are the calculated molecular masses of M_1-M_6. Endo is endostatin, NANA *N*-acetylneuraminic acid, and Gal-GalNAc galactose *N*-acetylgalactosamine.

compound (M_1) as a protein of 22 000 Da. Analyzing fractions 27 and 28, we unambiguously found further molecular masses of 21 841 Da (M_2), 21 710 Da (M_3), and 21 549 Da (M_4) (Table 1).

Carbohydrate Analysis. Using the DIG glycan differentiation kit for structure analysis of oligosaccharides of the endostatin-containing fractions (Figure 1C), positive spots could be observed for *N*-acetylneuraminic acid (NANA, α2−3 and α2−6) and galactose β-(1−3)-*N*-acetylgalactosamine [Gal-β(1−3)GalNAc] (Figure 3). When the human 18.5 kDa form of endostatin was tested, no positive reaction was observed, an indication of the lack of glycosylation (Figure 3).

The composition of fractions 28 and 29 was characterized (Figure 1D,E) before and after enzymatic treatment with exoglycosidases using HPLC−ESMS. Dependent on the enzymes used, the relative intensities of the detected *m*/*z* ratios belonging to the masses M_1-M_4 differed significantly (Table 1). After treatment of fraction 29 (M_1 = 22 000 Da) with neuraminidase followed by *O*-glycosidase, new molecular masses of 21 705 Da ± 0.02% (M_3) and 21 338 Da ± 0.02% (M_5) appeared. By hydrolyzing fraction 28 enzymatically, we could show that the ratio M_1/M_2 did not change within the tolerance before and after incubation independent of the added enzymes (Table 1). In contrast, the ratios M_1/M_3 and M_2/M_4 clearly decreased after incubation with neuraminidase and they increased after *O*-glycosidase treatment (Table 1). Furthermore, a substance with a molecular mass of about 21 338 Da ± 0.02% (M_5) was detectable, whereas a compound related to the calculated molecular mass of 21 187 Da ± 0.02% (M_6) could not be found in this sample. Evidence for M_6 was obtained after incubation of fraction 27 with neuraminidase and *O*-glycosidase. Taken together, the 21−22 kDa endostatins revealed glycosylation at T^{125} by Gal-GalNAc and additional NANA.

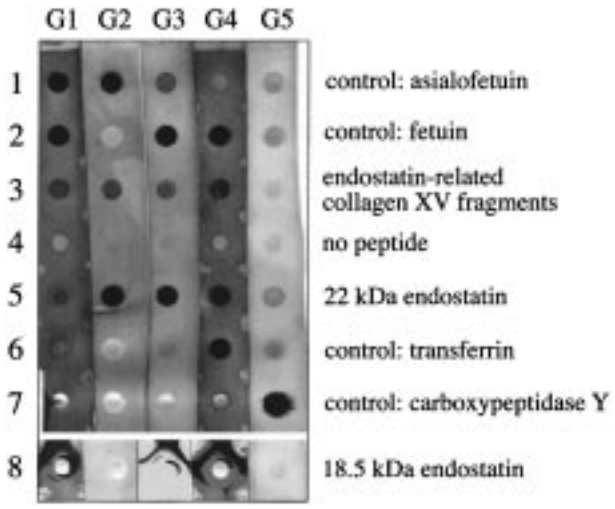


FIGURE 3: Carbohydrate analysis of the 22 kDa endostatins and the endostatin-related collagen XV fragments by glycan differentiation. The 22 kDa endostatin was taken from the penultimate purification step 4 according to Figure 1C. Endostatin-related collagen XV proteins were purified via a similar procedure corresponding to step 5 (Figure 1D). Proteins were immobilized onto nitrocellulose followed by incubation with lectin−digoxigenin conjugates, enabling staining with Ponceau S solution. For comparison, the 18.5 kDa endostatin (*24*) was analyzed in parallel. Dark spots represent positive reactions for the following glycans: G1, galactose β(1−4)-*N*-acetylglucosamine; G2, galactose β(1−3)-*N*-acetylgalactosamine; G3, *N*-acetylneuraminic acid (α2−3); G4, *N*-acetylneuraminic acid (α2−6); and G5, mannose.

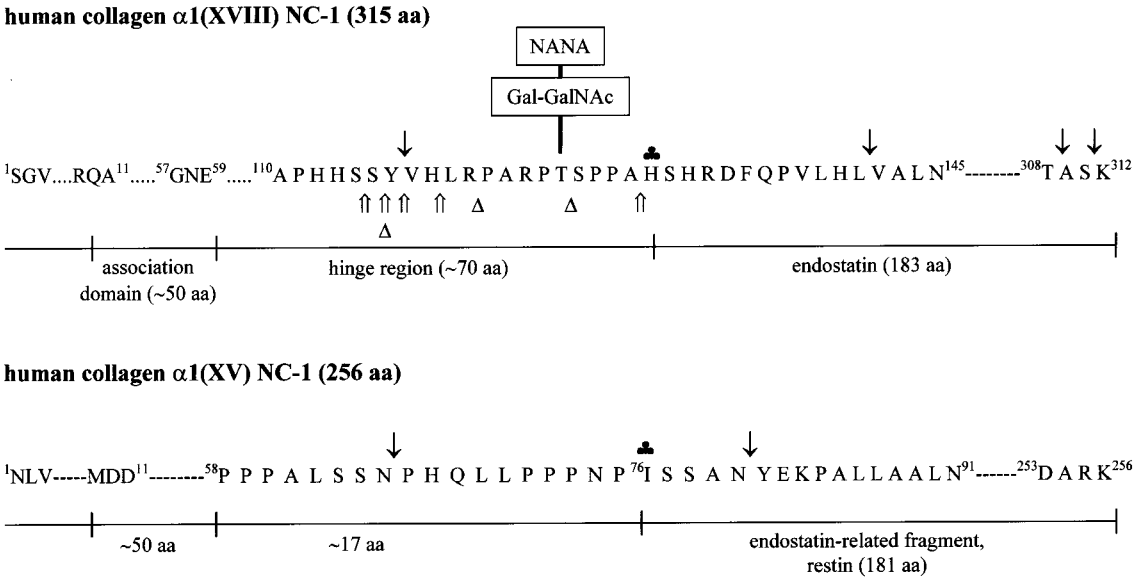


FIGURE 4: Schematic diagram of the human collagen XVIII and collagen XV NC-1 region. Proteolytic cleavage sites detected in collagen XVIII fragments (endostatins) and collagen XV fragments (restins) isolated from human plasma (Δ) and mouse hemangiomendothelioma (open upward arrow) (*11*) or human hemofiltrate (↓) (*24*) are denoted with arrows. The cloverleafs mark the start of the recombinant forms of murin endostatin (*11*) and human restin (*25*). The numbering of amino acids follows that described by Muragaki et al. (*12*), and subregions of the NC-1 domains in the entitled sites are numbered according to the system described by Rehn et al. (*32*). In vivo processing of the collagen XVIII NC-1 domain occurs in the hinge region, generating endostatin forms differing in length and status of glycosylation (NANA, *N*-acetylneuraminic acid; Gal-GalNAc, galactose *N*-acetylgalactosamine). Note the C-terminal deletion of one (lysine) or three amino acid residues, respectively. Comparable plasma proteolysis is observed for the human collagen XV NC-1 domain, producing circulating endostatin-related proteins.

Endostatin-Related Fragments of Collagen XV. During the purification of endostatin proteins from another charge of human hemofiltrate, fractions corresponding to the fourth purification step (Figure 1C) exhibited three additional immunoreactive bands with apparent masses of 22−26 kDa (Figure 2B). In comparison to the endostatin variants, these proteins eluted earlier on the reversed-phase columns. The proteins recognized by the anti-endostatin antibody mentioned above were purified to homogeneity by a procedure identical to that described for the endostatin polypeptides. CZE analyses of the purified fraction showed UV profiles comparable to those shown for endostatin in Figure 1E. N-Terminal sequencing of the first 20 amino acids yielded the two following major sequences: PHQLLPPPNPISSANYEKPALH and YEKPALHLAALNMPFSGDIR, corresponding to residues 66−87 and 81−100 of the human collagen XV NC-1 domain, respectively (*12*). The molecular masses of the collagen XV fragments were determined by ESMS, resulting in values of 20 995, 19 904, and 16 373 Da (±0.02% for all). With respect to their sequences and molecular masses, these collagen XV fragments are closely related to the NC-1 domain of collagen XVIII and could be designated as endostatin-related collagen XV proteins. The glycan differentiation test of the purified fraction containing the three endostatin-related collagen XV proteins showed positive reactions for NANA (α2−3 and α2−6), Gal-*β*(1−3)GalNAc, and Gal-*β*(1−4)GlcNAc (Figure 3, row 3).

DISCUSSION

Human hemofiltrate was recently shown to be a valuable source for the isolation of known and unknown bioactive peptides (*22*, *23*), and although hemofiltrate is obtained from patients with chronic renal failure, the main bulk of regulatory peptides is widely identical to that of normal subjects (*23*, *29*). This paper describes the purification of novel, glycosylated forms of endostatin from hemofiltrate as well as the isolation of circulating forms of endostatin-related collagen XV proteins. A five-step purification procedure and polyclonal antibodies directed against the N-terminal sequence of human 18.5 kDa endostatin (*24*) enabled us to isolate highly enriched endostatin forms, which differ in some amino acids or carbohydrate chains from each other, respectively. These compounds were classified by N-terminal sequence analysis as C-terminal fragments of human collagen XVIII, starting with an identical amino acid sequence (V^{117}HLR...), predicted to be a potential cleavage site of the NC-1 domain of human collagen XVIII (see Figure 4). C-Terminal sequencing of the major compound confirmed the identification as endostatin(s), lacking the final three or the terminal lysine residue in endostatin proteins isolated and characterized so far (*24*).

Mass determination of the endostatin forms using ESMS determined the major component to be a 22 000 Da form (M_1), while additional lower masses (M_2−M_4) could be detected, none of which could be correlated to a single amino acid sequence of endostatin. It can be concluded that different glycosylation patterns at residue 9 (T^{125}) are responsible for this heterogeneous distribution, which was proven by a glycan differentiation test. While carbohydrates including NANA (M_r = 291 g/mol) and galactose *N*-acetylgalactosamine (M_r = 365 g/mol) were identified for the major 22 kDa endostatin, no carbohydrate was found attached to the originally described 18.5 kDa form (*24*). With regard to the similarities in sequence, polarity, molecular mass, and extent of glycosylation, forms of circulating human endostatin were postulated as presented in Table 1. Moreover, endostatin forms also differ in their C-terminal sequence, since the shorter one (M_6, V^{117}−T^{309}) has lost the residues A^{310} and

S^{311} representing the C-terminus of the longer form (M_5, $V^{117}-S^{311}$). On the basis of these two endostatin sequences, a similar glycosylation is predicted for both molecules, including O-glycosylation at T^{125} (residue 9) with Gal-GalNAc (M_3 and M_4) and an additional linkage to NANA (M_1 and M_2). Experimental evidence of this expectation was found using HPLC−ESMS technology, allowing a reliable semiquantitative analysis of different protein forms in a prepurified mixture. The carbohydrate chains identified in endostatin can be classified as the mucin type with the usual Gal-GalNAc core unit of *O*-glycans (*30*). In many cases, this disaccharide is substituted by one or two sialic acid moieties as confirmed in this study. Forms of endostatin with a higher degree of glycosylation enlarging the core unit can be expected as indicated by the evidence of α(2−3)- and α-(2−6)-linked NANAs (see Figure 3). Whether these carbohydrate residues are derived from the parent collagen XVIII modified by exoglycosidases or whether they were added to the unglycosylated polypeptide core by the action of transferases remains to be determined. Nevertheless, the possibility of unspecific and/or nonenzymatic cleavage of NANA from the Gal-GalNAc residues during preparation and isolation of endostatin under acidic conditions cannot be excluded.

The physiological relevance of glycosylation at a "hot spot" of the parent collagen XVIII molecule is potentially interesting in terms of several as yet undefined properties of endostatin(s).

(a) The hydrophilic and polar charge distribution of glycans can increase the solubility of endostatin, and (b) sialic acid residues may protect the polypeptide from uncontrolled digestion by proteases or (c) may allow the sorting in the Golgi complex by affecting the protein conformation. Finally, (d) the direct interactions of endostatin(s) with putative cell receptors or binding proteins may depend on the degree of glycosylation as seen in other systems (*31*). Such differences in binding properties can be responsible for the currently unsolved large variations in the functional activities of endostatin(s) from different laboratories and sources. Taken together, glycosylation of endogenous endostatin may contribute to the release and action of active forms of the molecule related to (anti-) angiogenesis. Compared to the physiologically active, nonglycosylated murine analogue of endostatin (truncated N-terminus lacking the glycosylation site), the herein presented forms of human endostatin and the formerly described nonglycosylated 18.5 kDa molecule appear to be intermediates or products during proteolytic processing of the NC-l domain of collagen XVIII into active endostatin(s) and inactivate shorter forms (see Figure 4). Preliminary data did not show antiproliferative but significant antimigratory effects of the glycosylated endostatins on vessel wall cells (S. Kanse, unpublished results). Loss of natively chelated Zn^{2+} ions essential for antiangiogenic activity (*19*) due to chromatographic treatment with different pHs and organic solvents may affect the functional activities of these proteins.

On the basis of immunological cross reactivity with endostatin, novel circulating collagen XV-related C-terminal proteins were identified in hemofiltrate, and their subsequent characterization revealed a high degree of homology to collagen XVIII-derived endostatin(s). Both parent collagens have a similar distribution and may undergo comparable proteolytic processing in vivo. In fact, during the preparation of this report, a functional role for collagen XV-related protein(s), termed restin (*25*), was shown that relates to inhibitory effects on endothelial cell migration as well as an antiangiogenic activity in vivo using the endostatin-homologous recombinant 22 kDa collagen XV fragment. The masses determined by ESMS of the herein presented collagen XV fragments [20 995, 19 904, and 16 373 Da (±0.02% for all)] do not correlate with those calculated from the pure amino acid sequences (P^{66}HQLLPP...DARK^{256}, 21 271 Da; Y^{81}EKPALH...DARK^{256}, 19 707 Da). Therefore, we assume that C-terminal truncation and/or protein modifications such as glycosylation similar to that of endostatin account for the observed differences in molecular masses. Future studies will focus on the biological relevance of such glycosylated endostatin(s) and restin(s) in an effort to unravel their biological role in angiogenesis as related to glycosylation.

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