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Xylosyltransferase I acceptor properties of fibroblast growth factor and its fragment bFGF (1–24) ☆

Joachim Kuhn [a,*], Martina Schnölzer [b], Sylvia Schön [a], Sandra Müller [a], Christian Prante [a], Christian Götting [a], Knut Kleesiek [a]

[a] *Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Georgstraße 11, 32545 Bad Oeynhausen, Germany*
[b] *Zentrale Proteinanalytik B100, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany*

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Abstract

Human basic fibroblast growth factor (bFGF) is a heparin-binding growth factor containing a G-S-G-motif which is a potential recognition sequence of xylosyltransferase I (XT-I). Here, we show that the recombinant human bFGF was xylosylated in vitro by human XT-I and that the fragment bFGF (1–24) is a good XT-I acceptor ($K_m = 20.8$ μM for native XT-I and $K_m = 22.3$ μM for recombinant XT-I). MALDI and MALDI-PSD time-of-flight mass spectrometric analyses of the xylosylated bFGF protein demonstrate the transfer of xylose to the serine residue of the G-S-G-motif in the amino terminal end of bFGF. The peptide bFGF (1–24) is well suitable as an acceptor substrate for XT-I and can be used in a radiochemical assay to measure the XT-I activity in cell culture supernatant and human body fluids, respectively. Furthermore, we could demonstrate that the XT-I interacts strongly with heparin and that this glycosaminoglycan is a predominantly non-competitive inhibitor of the enzyme using the fragment bFGF (1–24) as xylose acceptor.

Keywords: Xylosyltransferase; Basic fibroblast growth factor; Heparin

The formation of proteoglycans is initiated by xylosyltransferase I (EC 2.4.2.26, XT-I) transferring xylose from UDP-xylose to consensus serine residues of proteoglycan core proteins. Only selected serine residues are recognized by the enzyme. Detailed investigations based on the alignment of the amino acid sequence of 51 known chondroitin sulfate attachment sites in unrelated proteoglycans suggested a recognition sequence composed of the amino acids a-a-a-a-G-S-G-a-b-a with a = E or D and b = G, E, or D [1,2]. Interestingly, the G-S-G-motif was found to occur in the flexible amino terminal end of the human basic fibroblast growth factor (bFGF). Xylosylated bFGF has not yet been detected in vivo, and existing analytical methods developed for the investigations of bFGF are not sensitive enough as they are only effective in the range of 10–100 ng of purified proteins/ml. However, the bFGF serum levels in humans are in the pg/ml range.

Proteoglycans, which are the major components of the extracellular matrix (ECM), show a particularly broad cooperation with cytokines and growth factors. Of great importance are the interactions of these ECM molecules with the members of the fibroblast growth

☆ *Abbreviations:* XT-I, xylosyltransferase-I; rXT-I-HIS, recombinant histidine-tagged XT-I; bFGF, basic fibroblast growth factor; bFGF (1–24), bFGF fragment PALPEDGGSGAFPPGHFKDPKRLY; DPBS, Dulbecco's phosphate-buffered saline; ECM, extracellular space; MALDI-TOF mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Mops, 3-(*N*-morpholino)propanesulfonic acid; PSD, post-source decay.

* Corresponding author. Fax: +49 5731 972013.
E-mail address: jkuhn@hdz-nrw.de (J. Kuhn).

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factor (FGF) family comprising of at least 12 heparin-binding proteins. Proteoglycans store the soluble factors in the matrix and release the growth factors upon digestion of heparan sulfate proteoglycans (HSPG) by glycosaminoglycan-degrading enzymes or protease activity [3–5]. HSPG protects bFGF from degradation and thermal denaturation [6]. Furthermore, the activity of bFGF, which is involved in many biological processes including cell proliferation, differentiation, angiogenesis, and wound healing, is mediated by binding to HSPG [7–10].

Here, we investigate for the first time bFGF and the amino terminal bFGF fragment (1–24) as potential in vitro substrates for the XT-I mediated xylosylation. In addition, the inhibitory effect of the glycosaminoglycan heparin on the XT-I activity is analyzed in detail.

Methods

Materials. The JAR choriocarcinoma cells were purchased from ATCC (Rockville, MD) and *High Five* insect cells derived from *Trichoplusia ni* were from Invitrogen (San Diego, CA). The human SAOS-2 osteogenic sarcoma cell line was obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the telomerase-immortalized hTERT-BJ1 cell line was from BD Biosciences Clontech (Heidelberg, Germany). Dried UltraDOMA-PF and Insect Xpress cell culture medium were obtained from BioWhittaker (Vervier, Belgium) and aqua ad injecta was from Braun (Melsungen, Germany). McCoy's 5A cell culture medium, Dulbecco's phosphate-buffered saline, antibiotic/antimycotic solution, heat-inactivated fetal calf serum, trypsin–EDTA solution, the Bicinchoninic Acid Protein Assay Kit, and the recombinant human bFGF were purchased from Sigma (Deisenhofen, Germany). DMEM cell culture medium was obtained from PAA Laboratories GmbH (Cölbe, Germany). Cell culture flasks, sterile pipettes, and tubes were purchased from Becton–Dickinson (Heidelberg, Germany). The bioreactor Tecnomouse was supplied by Integra Biosciences (Fernwald, Germany).

UDP-[^{14}C]-xylose (9.88 kBq/nmol) was purchased from DuPont (Bad Homburg, Germany), 25 mm diameter nitrocellulose discs were from Sartorius (Göttingen, Germany), and scintillation mixture and the liquid scintillation counter LS5000TD were supplied by Beckman (Fullerton, CA). The bFGF fragment PALPEDGGSGAFPPGHFK DPKRLY (bFGF (1–24)) from bovine brain was obtained from Bachem (Heidelberg, Germany). The chromatography medium POROS 20 HE, the C_{18} column (2.1 × 100 mm, 5-μm particle size), and the HPLC workstations Biocad Sprint and Integral were supplied by Perseptive Biosystems (Framingham, MA). Trypsin sequencing grade from bovine pancreas was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The MALDI mass spectrometer Reflex II was from Bruker Daltonik GmbH (Bremen, Germany). Precast polyacrylamide gels, buffers, and NuPAGE electrophoresis systems Xcell II Mini-Cell were from Novex (San Diego, CA). Microcon 3.000 ultrafiltration tubes were purchased from Amicon (Beverly, MA). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Serum collection. Venous blood samples were collected in serum separation tubes from KABE Labortechnik GmbH. After clotting and centrifugation, the serum was stored at −20 °C.

Preparation of XT-I. Native XT-I was secreted by JAR choriocarcinoma cells cultured in serum- and protein-free Ultradoma-PF medium supplemented with 1× antibiotic/antimycotic solution. A scale up of XT-I production was performed by cultivation of the cells in the hybrid hollow-fiber bioreactor Tecnomouse [11].

According to standard procedures [12], aliquots of cryoconserved JAR cells were thawed and plated in 175 cm^2 T-flasks containing Ultradoma-PF medium supplemented with 1× antibiotic/antimycotic solution and 10% heat-inactivated fetal calf serum. The cells were cultured at 37 °C in a humidified atmosphere (95% air and 5% CO_2). When confluency was nearly reached in the T-flasks, the adherent cells were removed using 0.05% trypsin and 0.02% EDTA in DPBS. After the cells were gently washed twice with DPBS and suspended in Ultradoma-PF medium, the cells were seeded in T-flasks with serum- and protein-free Ultradoma-PF medium supplemented with 1× antibiotic/antimycotic solution without fetal calf serum. The cells were cultured under serum-free conditions, and before confluency was reached, the adherent cells were removed during the exponential growth phase with 0.05% trypsin and 0.02% EDTA in DPBS by incubation at 37 °C for 10 min. After centrifugation (5 min, 1000*g*) of the cell suspension, the cell pellet was resuspended in 10 ml serum- and protein-free Ultradoma-PF medium and then inoculated into the ECS of the bioreactor Tecnomouse using a 10 ml syringe. The cells in the reactor were cultured using Ultradoma-PF medium at 37 °C in a humidified 5% CO_2/95% air atmosphere as described previously [11].

SAOS-2 and hTERT-BJ1 cell lines, which were also used in this study, were cultivated according to standard techniques [12]. The cell culture medium used for SAOS-2 cells was McCoy's 5A with 10% FCS and the medium used for hTERT-BJ1 cells was DMEM with 10% FCS. In addition, both media used were supplemented with 1× antibiotic/antimycotic solution and 2 mM L-glutamine. Cells were grown in 175-cm^2 T-flasks. After confluence was reached, the cell culture supernatant was harvested, and the XT-I activity and the protein concentration in the spent medium were measured by XT-I activity assay with bFGF (1–24) as xylose acceptor and bicinchoninic acid protein assay, respectively.

Cloning of human XT-I cDNA and overexpression of recombinant XT-I (rXT-I-HIS) were performed as described previously [13]. *High Five* insect cells containing the XT-I expression vector pCG255-1 (*High Five*/pCG255-1) were cultivated in roller bottles with serum-free insect Xpress cell culture medium containing 10 μg/ml gentamicin and 5 μg/ml blasticidin S at 27 °C by 12 rph. The cell density was maintained between 10^6 and 2.5×10^6 cells per milliliter. The rXT-I-HIS containing cell culture medium was harvested every 4 days after centrifugation of the cell suspension for 5 min at 1000*g*.

Heparin affinity chromatography. The interaction of native and recombinant XT-I with heparin was investigated using heparin affinity chromatography on POROS 20 HE. JAR cell culture supernatant from the hybrid hollow-fiber bioreactor as well as cell culture supernatant from *High Five*/pCG255-1 were passed through a 0.2-μm filter. In each case, 1.0 ml of the filtrate was applied to a POROS 20 HE column (4.6 × 100 mm) and equilibrated with buffer A (40 mM sodium acetate, pH 6.0) at a flow rate of 10 ml/min. The column was washed with 12 ml buffer A, and the proteins adsorbed were eluted with the same buffer containing NaCl by a linear gradient of 0.00–1.20 M NaCl (28 ml).

For XT-I acceptor studies, the native and recombinant XT-I was purified using heparin affinity chromatography on POROS 20 HE. 10.0 ml of cell culture supernatant from JAR cells or from *High Five*/pCG255-1 was passed through a 0.2-μm filter and applied to a POROS 20 HE column (16 × 100 mm) equilibrated with buffer A at a flow rate of 40 ml/min. After washing the column with 100 ml buffer A, the XT-I adsorbed protein was eluted with the same buffer containing NaCl. The NaCl concentration was increased stepwise: 20 ml buffer A, 0.09 M NaCl; 20 ml buffer A, 0.15 M NaCl; 30 ml buffer A, 0.24 M NaCl; 24 ml buffer A, 0.30 M NaCl; 24 ml buffer A, 0.60 M NaCl; 24 ml buffer A, 1.00 M NaCl; and 24 ml buffer A, 1.89 M NaCl. Fractions of 38 ml were collected, and the XT-I activity was measured. The XT-I containing fractions were pooled and used for further investigations.

XT-I activity assay. The method for determination of XT-I activity is based on the incorporation of [^{14}C]-D-xylose with a XT-I-acceptor [1,2]. Recombinant bikunin was expressed in *Escherichia coli* strain BL21(DE3) as described previously [1]. Bikunin (1.5 μM) or varying amounts of bFGF as well as bFGF fragments were incubated with XT-I solution from JAR choriocarcinoma cell culture supernatant or with rXT-I-HIS and UDP-[^{14}C]-D-xylose. The reaction mixture for the assay contained in a total volume of 100 μl: 50 μl of XT-I solution, 25 mM of 4-morpholineethanesulfonic acid (pH 6.5), 25 mM KF, 5 mM $MgCl_2$, 1.0 μM UDP-[^{14}C]-D-xylose, and varying XT-I acceptor concentrations. When the interaction of XT-I and heparin was investigated, 0.2 μg LMW-heparin (MW = 6.000 Da) was added to the reaction mixture. After incubation for 1 h at 37 °C, the reaction mixtures were placed on nitrocellulose discs. After drying, the discs were washed for 10 min with 10% trichloroacetic acid and three times with 5% trichloroacetic acid solution. Incorporation radioactivity was measured by liquid scintillation counting. The enzyme activity was expressed in units (1 U = 1 μmol of incorporated xylose per min).

Analysis of xylosylated bFGF fragment (1–24). XT-I incubation mixtures of bFGF fragment (1–24) were fractionated by reversed-phase chromatography on a C_{18} column (2.1 × 100 mm, 5-μm particle size). After equilibration with 0.1% (vol/vol) trifluoroacetic acid, 100 μl of sample solution was loaded onto the column. The column was eluted at room temperature with a 27-min gradient from 0% to 50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.4 ml/min. Fractions of 1 ml were collected, lyophilized, and used for MALDI mass spectrometric analysis.

Analysis of bFGF after in vitro xylosylation. For in vitro xylosylation of the recombinant growth factor, 73.2 nM (12 μg) bFGF was incubated at 37 °C for 4 h in a reaction mixture (25 μl) containing 25 mM Mes, pH 6.5, 25 mM KCl, 5 mM KF, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 31.3 μU heparin affinity purified XT-I, and 37 mM UDP-D-xylose. A reaction mixture without UDP-D-xylose was used as a control. After incubation, the reaction mixtures were diluted 1:5 with aqua destillata and purified by SDS–PAGE as follows. 12.1 μl of sample was added to 4.7 μl sample buffer containing 1.17 M sucrose, 1.00 M Tris/HCl, pH 8.5, 0.28 M SDS, 2.08 mM EDTA, 0.88 mM Serva blue G250, 0.70 M phenol red, and 0.10 M dithiothreitol. The mixtures were heated for 10 min at 99 °C and, after the samples had been loaded, SDS–polyacrylamide gel electrophoresis was carried out on a 4–12% Bis-Tris polyacrylamide gel with Mops running buffer (50 mM Mops, 50 mM Tris, 3.47 mM SDS, and 1.03 mM EDTA, pH 7.7). Protein bands were detected by Coomassie brilliant blue staining, and the bFGF bands were excised and characterized by MALDI mass spectrometry.

MALDI and MALDI-PSD mass spectrometry. Coomassie-stained bFGF was excised from the gel, repeatedly washed with H_2O and H_2O/acetonitrile, and digested overnight with trypsin at 37 °C. The peptides generated in the supernatant were analyzed by MALDI mass spectrometry.

Sample preparation was achieved by cocrystallization of matrix with ZipTip C18 (Millipore) concentrated samples. Briefly, the peptides in the supernatant of the in-gel digestion were absorbed to a prewashed (50% acetonitrile/water) and equilibrated (using 0.1% trifluoroacetic acid/water) Zip Tip C18 by repetitive pipetting steps. Following washing of the Zip Tip C18 by equilibration buffer, the peptides were eluted from the Zip Tip with 1 μl matrix (α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile/water).

MALDI mass spectra were recorded in the positive ion mode with delayed extraction on a Reflex II time-of-flight instrument equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. Ion acceleration voltage was set to 26.5 kV, the reflector voltage was set to 30.0 kV, and the first extraction plate was set to 20.6 kV. Mass spectra were obtained by averaging 50–200 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at *m/z* 842.50 and *m/z* 2211.10.

Post-source decay (PSD) analysis was performed in the positive ion reflector mode with delayed extraction by setting an ion gate width of 40 Da around the ion of interest. Data were acquired in 14 segments by decreasing the reflector voltage in a stepwise fashion. For each segment, 200 individual laser shots were accumulated. The fragment ion spectrum was obtained by pasting together all segments to a single spectrum using the FAST software provided by Bruker. Fragment ion calibration was performed externally with the fragment masses of the adrenocorticotropic hormone (ACTH) 18–39 clip.

Singly charged monoisotopic peptide masses were used for database searching. Searches were performed against the NCBInr database using the ProFound search algorithm (http://129.85.19.192/prowl-cgi/ProFound.exe) and the Protein prospector software developed at the University of California, San Francisco (http://prospector.ucsf.edu), with an IEP range from 0 to 14 and the oxidation of methionine as possible modification. Up to one missed tryptic cleavage site was considered, and the mass tolerance for the monoisotopic peptide masses was set to ±100 ppm.

Searches with fragment masses from PSD experiments were performed against the NCBInr database using the MS-Tag search algorithm provided by the Protein prospector software package. Parent mass tolerance was set to ±0.1 Da and fragment ion tolerance was set to ±0.7 Da.

Trypsin digestion of bFGF for the investigation as XT-I acceptor substrate. A sample of 3 μg bFGF was digested with 0.3 μg trypsin sequencing grade at 37 °C for 2 h according to the method recommended by the manufacturer. After incubation, the sample was heated to 99 °C for 10 min to denature the trypsin. The sample was then assayed in the XT-I activity assay.

Measurement of protein concentration. The total protein concentration was determined with the Bicinchoninic Acid Protein Assay Kit using bovine serum albumin as standard. Free amino acids in the samples were removed prior to protein determination by ultrafiltration with Microcon 3.000 tubes according to the manufacturer's instructions.

Results

bFGF and the fragment bFGF (1–24) as potential XT-I acceptors

We tested the recombinant human bFGF and the fragment bFGF (1–24) containing the consensus XT-I acceptor sequence as potential XT-I acceptors using purified native XT-I. Both protein and peptide showed XT-I acceptor properties using the in vitro XT-I activity assay. The affinity of XT-I for both acceptors was compared by determination of the Michaelis–Menten constants (K_m) for the xylosylation (Table 1). XT-I showed the highest activity for the fragment bFGF (1–24). The amino terminal fragment bFGF (1–18) should be released by tryptic digestion of bFGF (Fig. 1A). After

Table 1
Michaelis-Menten (K_m) constants for the xylosylation of bFGF and bFGF fragment (1–24) by xylosyltransferase I

Enzyme	Acceptor	K_m (μM)
Native XT-I	bFGF	57.2
Native XT-I	bFGF fragment (1–24)	20.8
rXT-I-HIS	bFGF fragment (1–24)	22.3

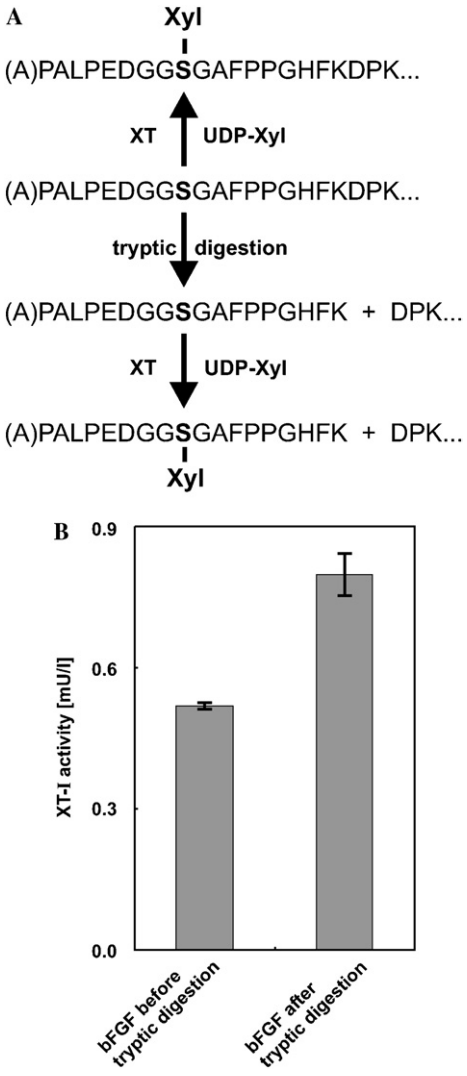

Fig. 1. The bFGF and the tryptically digested bFGF as XT-I acceptor. (A) Schematic representation of the xylosylation of bFGF by XT-I before and after tryptic digestion. The first amino acid "alanine" shown in brackets is from the vector construction. (B) Increased incorporation of xylose was measured after tryptic cleavage of the bFGF protein. Results are means ± SE of two different experiments.

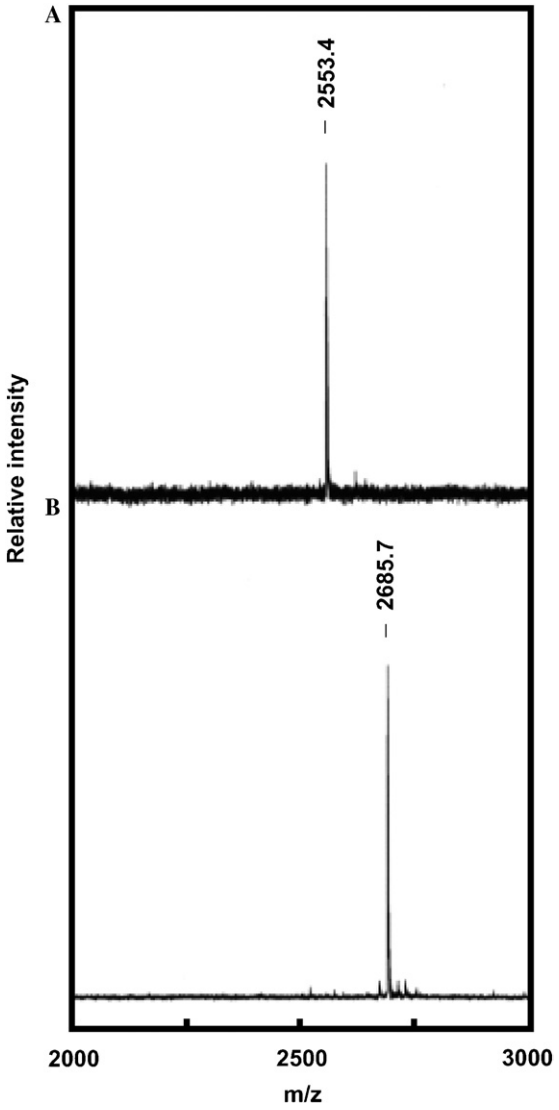

Fig. 2. Mass spectrometric analysis of the xylosylated peptide bFGF (1–24). (A) MALDI-TOF mass spectrum of the untreated peptide bFGF (1–24) [$m/z = 2553.4$] and (B) the HPLC-purified peptide after XT-I treatment [$m/z = 2685.7$]. The difference of 132.3 corresponds to the mass of a xylosyl residue.

cleavage of bFGF with trypsin, the xylosylation of the protein fragments increased in comparison with intact protein (Fig. 1B).

Furthermore, we were able to measure the XT-I activity in serum and other human fluids with sufficient sensitivity (see below) by replacing the recombinant human bikunin which was previously used as XT-I acceptor in the radiochemical assay [1,2] with fragment bFGF (1–24) at a concentration of 10 μM.

Mass spectrometric analysis of the xylosylated fragment bFGF (1–24)

The xylosylation of the fragment bFGF (1–24) after XT-I treatment was investigated using MALDI mass spectrometry. Reaction mixtures of the XT-I assay with and without UDP-D-xylose as XT-I donator were subjected to RP-HPLC to separate the peptides from impurities, and the collected peptide fractions were used for MALDI analysis. Comparison of the MALDI-TOF spectra revealed that in the sample with UDP-D-xylose the m/z ratio of the bFGF peptide was increased from 2553.4 to 2685.7 Da (Fig. 2). The difference of 132.3 Da is equivalent to the mass of one xylose residue attached to the single serine residue of the bFGF peptide.

Mass spectrometric analysis of the xylosylated bFGF

The modification of the recombinant bFGF was investigated after treatment with XT-I. For carbohy-

drate analysis of the bFGF the protein was separated from the reaction mixture using SDS–PAGE. After tryptic digestion of the excised bFGF-Coomassie stained bands, the peptides obtained were analyzed with MALDI mass spectrometry. bFGF from the reaction mixture without UDP-D-xylose as XT-I donator was also separated by SDS–PAGE and the excised, tryptically digested bFGF-Coomassie-stained bands were

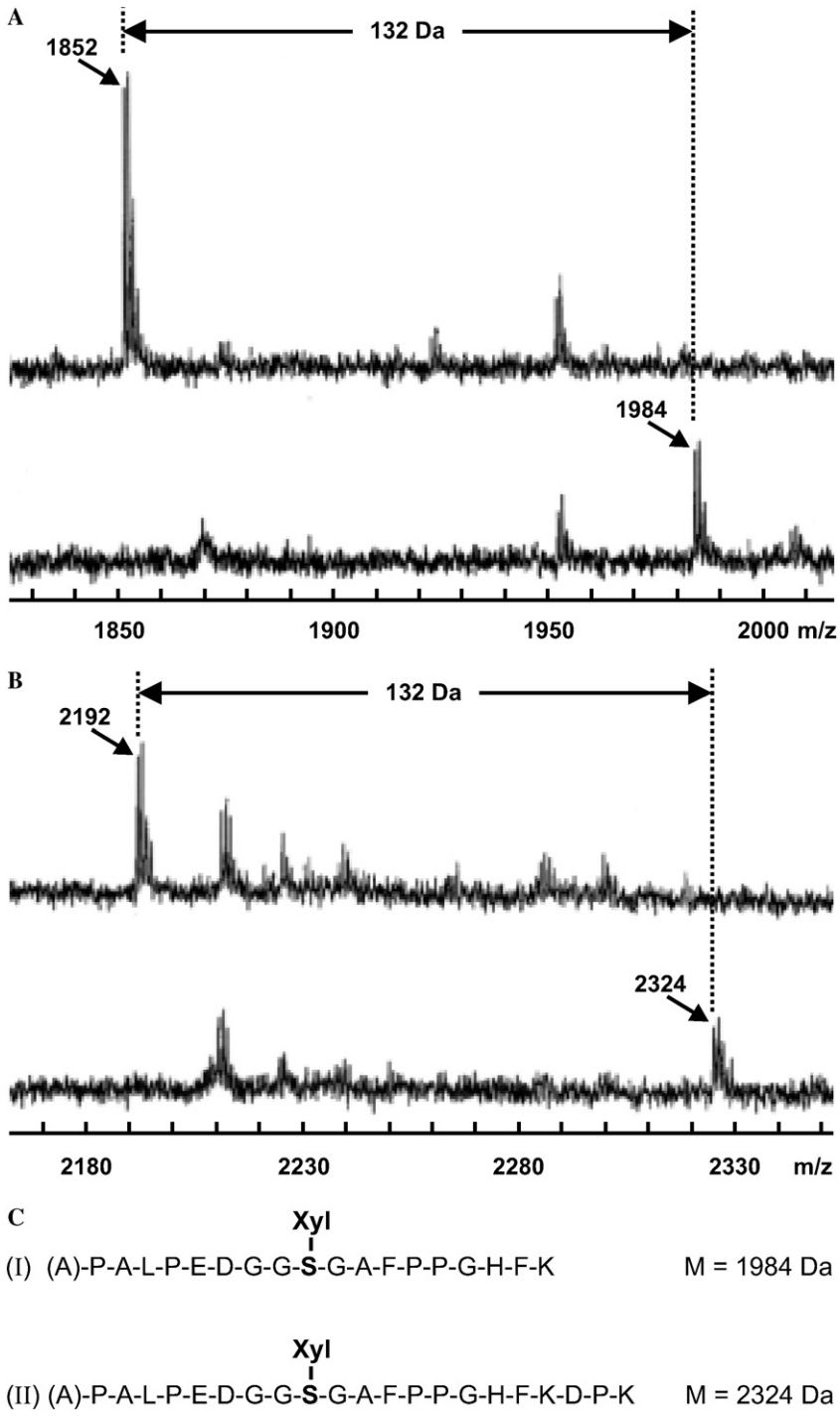

Fig. 3. Mass spectrometric analysis of the xylosylated bFGF. (A) Upper panel: the section of the mass spectrum of tryptically digested bFGF with a peptide fragment at *m*/*z* 1852 is displayed. Lower panel: the corresponding section of xylosylated bFGF shows no fragment at *m*/*z* 1852 but a fragment with *m*/*z* 1984. The mass difference of 132 Da between both fragments corresponds to the molecular mass of xylose. (B) Upper panel: another section of the mass spectrum of tryptically digested bFGF with a peptide fragment at *m*/*z* 2192 is displayed. Lower panel: the corresponding section of xylosylated bFGF shows no fragment at *m*/*z* 2192 but a fragment at *m*/*z* 2324. The mass difference of both fragments (132 Da) corresponds to the molecular mass of xylose. (C) Amino acid sequence of tryptic fragments of bFGF which correspond to ion signals at (I) *m*/*z* 1852 and with (II) *m*/*z* 2192. The first amino acid "alanine" shown in brackets is from the vector construction. The potential xylosylable serine is marked bold.

used as control. The MALDI spectrum of the peptides from the xylosylated bFGF shows two peptides with a mass increase of 132 Da in comparison to the peptides from the control, indicating the covalent attachment of a xylosyl group to these peptides (Fig. 3). However, the tryptic masses measured did not match the masses expected based on the amino acid sequence of the bFGF and xylosylated bFGF, respectively. To obtain the amino acid sequence of these peptides, MALDI-PSD fragment ion analysis was employed. The MALDI-PSD spectrum of the non-xylosylated peptide with m/z obtained after tryptic digestion of the growth factor is shown in Fig. 4. The parent ion at m/z 1851.9 was chosen for MALDI-PSD analysis, which (by sets of consecutive b- and y-ion signals) unambiguously confirmed the amino terminal sequence of the bFGF with an additional alanine at the N-terminal end. This investigation revealed that the glycosylated peptide from the xylosylated bFGF with m/z 1983.9 was the tryptically released end terminal sequence of the modified bFGF, and that the serine of the G-S-G-motif in the flexible amino terminal end of the bFGF was xylosylated by XT-I in vitro. The second xylosylated peptide with a m/z 2324.1 generated from the XT-I treated bFGF supports this result, because this glycosylated peptide was released by tryptic cleavage of bFGF after the second lysine of the amino terminal part (Fig. 3C).

K_m-values of native and recombinant XT-I

The bFGF fragment PALPEDGGSGAFPPGHFK DPKRLY was used for the investigation of the kinetic mechanisms of XT-I mediated xylosylation. The K_m-values of the native XT-I and of the recombinant XT-I with respect to the peptide concentration were determined as 20.8 and 22.3 μM, respectively (Table 1).

Heparin is a predominantly non-competitive inhibitor of XT-I

A direct interaction of XT-I with heparin was demonstrated by heparin affinity chromatography on POROS 20 HE using the native enzyme from JAR choriocarcinoma cells cultured in the hollow-fiber bioreactor Tecnomouse and the rXT-I-HIS expressed in *High Five* insect cells. In each case, the XT-I activity was completely retained on the heparin matrix, and at least 0.5 M NaCl in the mobile phase was required to elute the enzyme (Fig. 5). The recombinant XT-I shows a stronger interaction with the heparin matrix (Fig. 5B, arrow) than the native enzyme (Fig. 5A, arrow). The interaction of XT-I and heparin was investigated with the XT-I activity assay using rXT-I-HIS, LMW-heparin (MW = 6.000 Da) from bovine and bFGF fragment PALPEDGGSGAFPPGHFKDPKRLY as substrate. XT-I activity was strongly inhibited by heparin and the kinetic study revealed that this inhibition was predominantly of the non-competitive type (Fig. 6). The apparent substrate inhibition constant (K_i) was determined to be 0.1 μM.

Quantification of XT-I activities in human serum and cell culture supernatant using bFGF fragment (1–24) as xylose acceptor

We determined XT-I activities in the serum samples of four patients with osteoarthritis. Similar results was found regardless of whether recombinant bikunin or

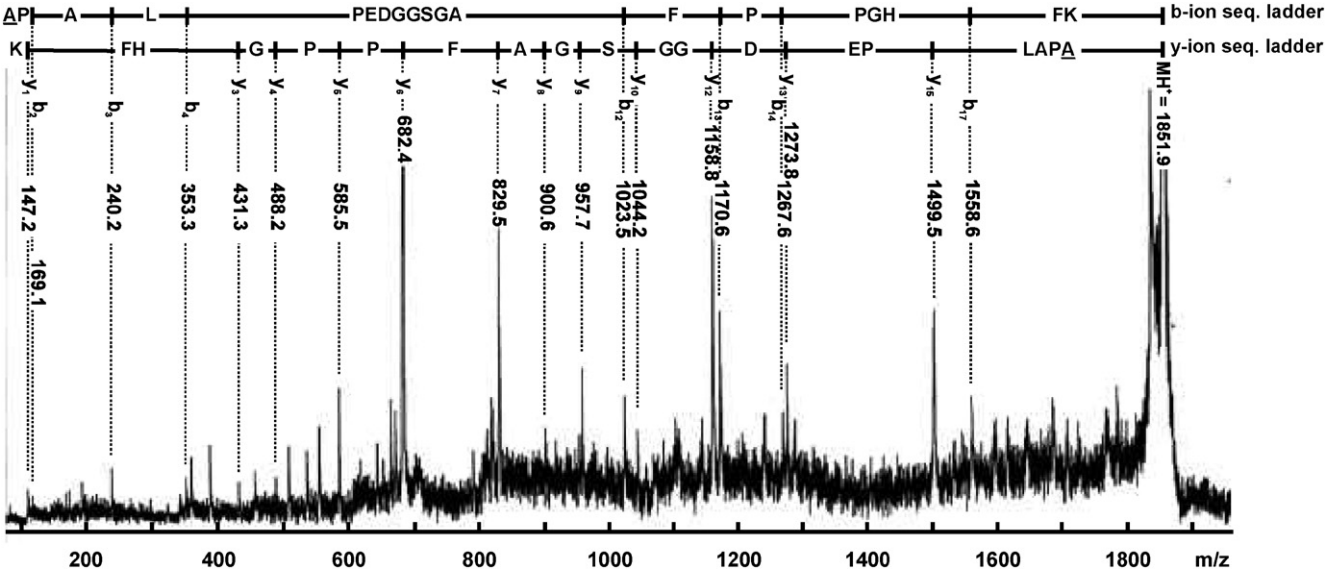

Fig. 4. PSD fragment ion spectrum of the tryptic bFGF peptide at m/z 1852. The MALDI-PSD fragments unambiguously show (by sets of consecutive b- and y-ion signals) the amino terminal sequence of bFGF with an additional alanine (underlined) at the extreme end.

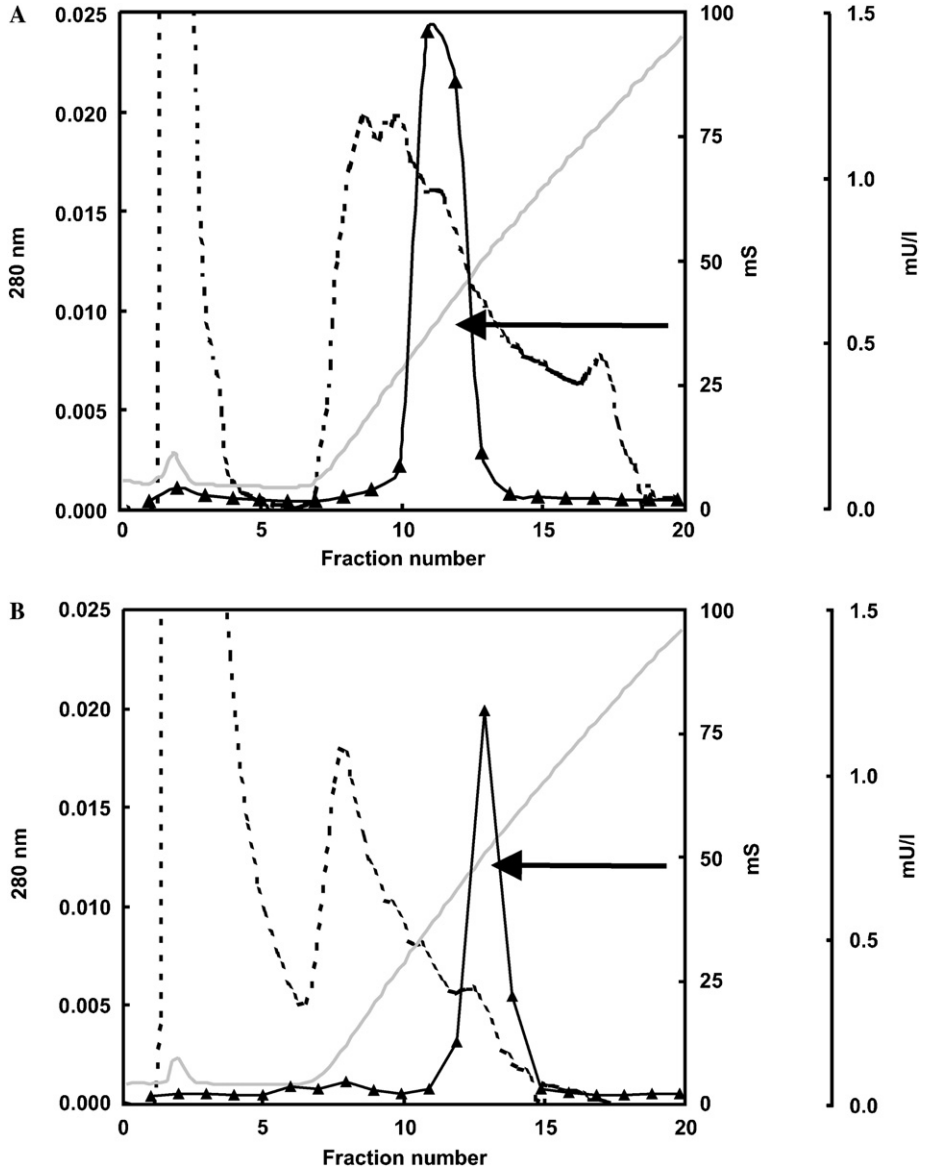

Fig. 5. Heparin affinity chromatography. A direct interaction of XT-I with heparin is demonstrated by heparin affinity chromatography using the native enzyme from JAR choriocarcinoma cells (A) and the recombinant XT-I from *High Five*/pCG255-1 (B). The XT-I sample was applied to a POROS 20 HE column. After washing with buffer A, the column was eluted with a linear NaCl gradient (gray line). Protein elution was monitored at A_{280} (dotted line), and fractions of 2.0 ml were assayed for XT-I activity (black triangles). In each case, XT-I activity was completely retained on the heparin matrix, and at least 0.5 M NaCl (ca. 35 mS) in the mobile phase was required to elute the enzyme. The recombinant XT-I shows a stronger interaction with the heparin matrix (arrow in B) than the native enzyme (arrow in A).

bFGF fragment (1–24) was used as xylose acceptor in the XT-I activity assay (Figs. 7A and B). All XT-I activities were within the normal range (0.8–1.5 mU/L) [2]. Furthermore, secretion of XT-I into extracellular space was measured for three human cell lines using bFGF fragment (1–24) as xylose acceptor (Fig. 7C). Highest XT-I activity was measured in the cell culture supernatant of JAR choriocarcinoma cells with 165 μU/g protein. This cell line secreted six times more XT-I than the osteogenic sarcoma cell line SAOS-2 with 26 μU/g protein and 40 times more XT-I than the fibroblast cell line hTERT-BJ1 with 4 μU/g protein.

Discussion

In the present study, we have xylosylated bFGF and the amino terminal bFGF fragment PALPEDGGSG AFPPGHFKDPKRLY in vitro using xylosyltransferase-I (XT-I). In vivo this enzyme is involved in the biosynthesis of the common carbohydrate-protein linkage structure of chondroitin sulfate, heparan sulfate, and dermatan sulfate proteoglycans. The in vivo substrate of XT-I is not a single well-defined compound, but rather a wide spectrum of different proteins containing an XT-I recognition sequence with the GSG-motif as

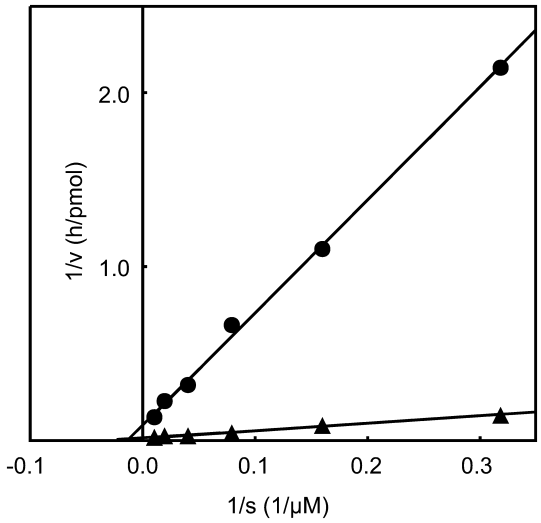

Fig. 6. Inhibition of XT-I activity by heparin. The Lineweaver–Burk plot shows the human XT-I activity (filled circles) and the strong inhibition of the enzyme activity after adding of heparin at 0.2 μg/ml to the reaction mixtures (filled triangles). This inhibition was predominantly of the non-competitive type and the apparent substrate inhibition constant (K_i) was determined to be 0.1 μM.

central unit. The bFGF is probably no in vivo substrate of XT-I although the growth factor contains the GSG-motif in its flexible amino terminal end. We have shown that the protein is xylosylated ($K_m = 57.2$ μM) in vitro and that in addition the bFGF fragment PALPEDGGSGAFPPGHFKDPKRLY is a good substrate for the XT-I (K_m for the native and the recombinant XT-I was 20.8 and 22.3 μM, respectively). XT-I activity could be measured in human serum and other body fluids with sufficient sensitivity with a modified assay which used the fragment bFGF (1–24) as XT-I acceptor substrate instead of recombinant human bikunin as described previously [1,2]. Using the fragment bFGF (1–24) as acceptor substrate for XT-I the troublesome and time-consuming synthesis of recombinant human bikunin can be avoided.

Xylosylation of the specific serine residues in the core protein is performed in the pre-Golgi compartment [14,15] but the majority of XT-I is found to be secreted into the extracellular space together with proteoglycans [16,17], whereas the other glycosyltransferases are mostly retained in the Golgi apparatus. The biological role of XT-I in the extracellular matrix is not yet understood. The occurrence of UDP-xylose in extracellular fluids is also not known to date and has to be investigated in further studies. Detection of extracellular UDP-xylose or other nucleotide sugars is generally limited by the lack of sensitive assays. Recently, a sensitive and specific assay for UDP-glucose mass was developed, and using this test, a clear observation of the release of UDP-glucose into the extracellular space as well as the function of this nucleotide sugar as a signaling molecule

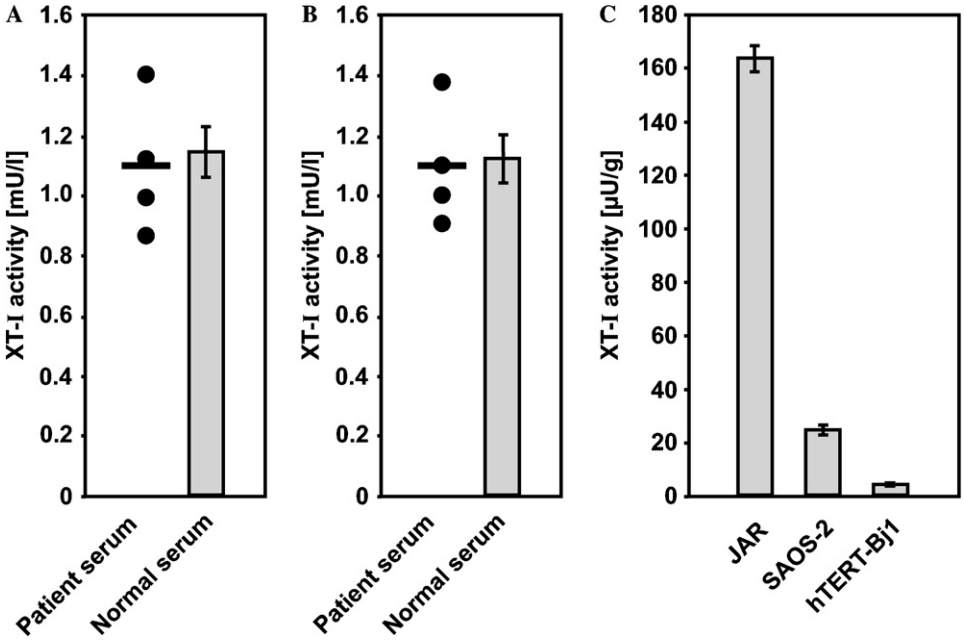

Fig. 7. XT-I activity in the serum of patients with osteoarthritis measured with a radiochemical assay using bikunin as xylose acceptor (A) and fragment bFGF (1–24) as xylose acceptor (B), respectively. Normal serum was obtained by mixing of the serum of 20 blood donors. Mean values are indicated by the bold line. XT-I activities in the cell culture supernatant of three different human cell lines (C). XT-I activity was determined in the cell culture supernatant when confluency was reached in the T-flask using the fragment bFGF (1–24) as xylose acceptor in the XT-I activity assay. Values given were measured in duplicate. Error bars indicate standard deviation. JAR, placental choriocarcinoma; SAOS-2, osteogenic sarcoma; hTERT-Bj1, telomerase-immortalized fibroblast cell line.

was reported [18]. The development of similar sensitive and specific assays for other nucleotide sugars especially for the measurement of UDP-xylose are of great interest.

In agreement with previous reports [19,20] we have shown a strong interaction of XT-I with heparin using heparin affinity chromatography. The native XT-I as well as the recombinant XT-I was completely retained on the heparin matrix, and a high salt concentration was required to elute the enzymes. The stronger interaction of the recombinant protein with the heparin in comparison with the native enzyme is most likely a result of the additional interaction of the histidine tail of the rXT-I-HIS. Heparin and heparan sulfate are linear and polysulfated polysaccharides known as glycosaminoglycan components of the extracellular matrix forming connective tissues in all animals. Both glycosaminoglycans interact with a variety of growth-associated molecules including midkine [21], pleiotrophin [22], and bFGF [23–29]. The heparin-binding domains of bFGF were indicated by analysis of the crystal structure of bFGF in the presence of a homogeneous heparin hexasaccharide [30]. Heparin sulfate oligosaccharides that bind bFGF have been found to be rich in IdoA(2S) containing disaccharides, and the affinity increased with chain length. The interaction of bFGF with heparin or heparan sulfate protects the growth factor against heat or acid denaturation and protease cleavage [31,29]. Here we have shown that the XT-I, which was secreted together with proteoglycans into the extracellular space, was able to xylosylate bFGF, and that the enzyme was strongly inhibited by heparin, suggesting that this glycosaminoglycan protects the growth factor from xylosylation. The 18 kDa form of bFGF is found outside the cell, although bFGF lacks a consensus signal sequence for secretion [32]. bFGF does not progress the regular secretory pathway through the endoplasmic reticulum and Golgi apparatus where xylosylation takes place. The mechanism of secretion of bFGF remains unclear, and it has been suggested that the growth factor is released from cells by cell damage, death, and non-lethal membrane disruptions [33]. Released bFGF is found stored in the ECM, and the distribution of the growth factor within the ECM is controlled by heparan sulfate proteoglycans [34].

The soluble form of recombinant bFGF is composed entirely of β-sheet structures as determined by multidimensional heteronuclear magnetic resonance microscopy [35]. Here, we demonstrated that the cleavage of bFGF generated a protein fragment pool which shows better XT-I substrate features than the intact protein, suggesting that bFGF was also protected against xylosylation by its three-dimensional structure. In addition, the good substrate properties of the bFGF fragment PALPEDGGSGAFPPGHFKDPKRLY indicate that xylosylation was performed on the GSG-motif of the flexible amino terminal end of the growth factor. This was clearly confirmed by our investigations of bFGF and xylosylated bFGF using MALDI mass spectrometry (Fig. 3) and MALDI-PSD analysis, which showed that the recombinant protein used had an additional alanine at the extreme end of the amino terminal sequence (Fig. 4).

Human and bovine bFGF are extremely highly conserved, suggesting that there is a very strong selective pressure to maintain its biological function. Only two amino acids at the carboxyl terminal end of the protein are different between the two species, giving an overall amino acid sequence homology of 99% [36]. bFGF is a motogenic, angiogenic, and survival factor involved in cell migration, cell differentiation, and a variety of development processes. Many biological functions of the growth factor are probably isoform-specific. In vivo xylosylation of bFGF could interfere with its biological function but, in the present study, endogenous bFGF xylosylation could not be measured because of the very low serum levels of bFGF. However, our investigations have shown the protection of the growth factor by its three-dimensional structure and by extracellular matrix components like heparin. The effects of post-translationally modified bFGF were demonstrated in previous studies [37–42]. It was shown that particularly non-enzymatic glycation of arginine and lysine residues in bFGF resulted in a weaker endothelial cell chemoattraction in vitro and weaker angiogenetic factor properties in vivo as compared to those of unmodified bFGF [39]. Further, it was reported that glycation reduced the mitogenic activity of bFGF in vitro [40], and that heparin and heparan sulfate protect basic fibroblast growth factor from non-enzymatic glycosylation [41].

In conclusion, our results clearly demonstrate that XT-I, an enzyme that is present in the extracellular space, xylosylates a specific serine residue at the amino terminal end of the recombinant human bFGF in vitro, and that the extracellular matrix molecule heparin is a predominantly non-competitive inhibitor of the enzyme. It remains to be elucidated whether in vivo xylosylation of bFGF also takes place and whether this post-translational modification interferes with the function of the growth factor. Finally, we have found that the commercially available fragment bFGF (1–24) with its single definite serine is an appropriate substrate for XT-I which can be used to measure the XT-I activity in cell culture supernatant, human serum, and other body fluids.

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References

[1] T. Brinkmann, C. Weilke, K. Kleesiek, Recognition of acceptor proteins by UDP-D-xylose proteoglycan core protein β-D-xylosyltransferase, J. Biol. Chem. 272 (1997) 11171–11175.
[2] C. Weilke, T. Brinkmann, K. Kleesiek, Determination of xylosyltransferase activity in serum with recombinant human bikunin as acceptor, Clin. Chem. 43 (1997) 45–51.
[3] P. Bashkin, S. Doctrow, M. Klagsbrun, C.M. Svahn, J. Folkman, I. Vlodavsky, Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules, Biochemistry 28 (1989) 1737–1743.
[4] J.A. Buczek-Thomas, M.A. Nugent, Elastase-mediated release of heparan sulfate proteoglycans from pulmonary fibroblast cultures. A mechanism for basic fibroblast growth factor (bFGF) release and attenuation of bfgf binding following elastase-induced injury, J. Biol. Chem. 274 (1999) 25167–25172.
[5] O. Saksela, D.B. Rifkin, Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity, J. Cell Biol. 110 (1990) 767–775.
[6] A. Yayon, M. Klagsbrun, J.D. Esko, P. Leder, D.M. Ornitz, Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor, Cell 64 (1991) 841–848.
[7] M. Fannon, M. Nugent, Basic fibroblast growth factor binds its receptors, is internalized, and stimulates DNA synthesis in Balb/c3T3 cells in the absence of heparan sulfate, J. Biol. Chem. 271 (1996) 17949–17956.
[8] D. Moscatelli, High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells, J. Cell. Physiol. 131 (1987) 123–130.
[9] A. Rapraeger, A. Krufka, B. Olwin, Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation, Science 252 (1991) 1705–1708.
[10] G. Sperinde, M. Nugent, Heparan sulfate proteoglycans control intracellular processing of bFGF in vascular smooth muscle cells, Biochemistry 37 (1998) 13153–13164.
[11] J. Kuhn, K. Mölle, T. Brinkmann, C. Götting, K. Kleesiek, High-density tissue-like cultivation of JAR choriocarcinoma cells for the in vitro production of human xylosyltransferase, J. Biotechnol. 103 (2003) 191–196.
[12] M. Butler, M. Dawson, Cell Culture Labfax, Bios Scientific Publishers (Academic Press), Oxford, England, 1992.
[13] J. Kuhn, S. Müller, M. Schnölzer, T. Kempf, S. Schön, T. Brinkmann, M. Schöttler, C. Götting, K. Kleesiek, High-level expression and purification of human xylosyltransferase I in High Five insect cells as biochemically active form, Biochem. Biophys. Res. Commun. 312 (2003) 537–544.
[14] A.E. Kearns, S.C. Campbell, J. Westley, N.B. Schwartz, Initiation of chondroitin sulfate biosynthesis: a kinetic analysis of UDP-D-xylose: core protein beta-D-xylosyltransferase, Biochemistry 30 (1991) 7477–7483.
[15] A.E. Kearns, B.M. Vertel, N.B. Schwartz, Topography of glycosylation and UDP-xylose production, J. Biol. Chem. 268 (1993) 11097–11104.
[16] C. Götting, S. Sollberg, J. Kuhn, C. Huerkamp, T. Brinkmann, T. Krieg, K. Kleesiek, Serum xylosyltransferase: a new biochemical marker of the sclerotic process in systemic sclerosis, J. Invest. Dermatol. 112 (1999) 919–924.
[17] H. Kähnert, R. Paddenberg, K. Kleesiek, Simultaneous secretion of xylosyltransferase and chondroitin sulfate proteoglycans in chondrocyte culture, Eur. J. Clin. Chem. Clin. Biochem. 29 (1991) 624–625.
[18] E.R. Lazarowski, D.A. Shea, R.C. Boucher, T.K. Harden, Release of cellular UDP-glucose as a potential extracellular signaling molecule, Mol. Pharmacol. 63 (2003) 1190–1197.
[19] K. Kleesiek, R. Reinards, J. Okusi, B. Wolf, H. Greiling, UDP-D-xylose: proteoglycan core protein β-D-xylosyltransferase: a new marker of cartilage destruction in chronic joint, Clin. Chem. Clin. Biochem. 25 (1987) 473–481.
[20] J. Kuhn, C. Götting, M. Schnölzer, T. Kempf, T. Brinkmann, K. Kleesiek, First isolation of human UDP-D-xylose: proteoglycan core protein β-D-xylosyltransferase secreted from cultured JAR choriocarcinoma cells, J. Biol. Chem. 276 (2001) 4940–4947.
[21] T. Kojima, A. Katsumi, T. Yamazaki, T. Muramatsu, T. Nagasaka, K. Ohsumi, H. Saito, Human ryudocan from endothelium-like cells binds basic fibroblast growth factor, midkine, and tissue factor pathway inhibitor, J. Biol. Chem. 271 (1996) 5914–5920.
[22] T.A. Mitsiadis, M. Salmivirta, T. Muramatsu, H. Muramatsu, H. Rauvala, E. Lehtonen, M. Jalkanen, I. Thesleff, Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial–mesenchymal interactions during fetal development and organogenesis, Development 121 (1995) 37–51.
[23] P. Jemth, J. Kreuger, M. Kusche-Gullberg, L. Sturiale, G. Gimenez-Gallego, U. Lindahl, Biosynthetic oligosaccharide libraries for identification of protein-binding heparan sulfate motifs. Exploring the structural diversity by screening for fibroblast growth factor (FGF)1 and FGF2 binding, J. Biol. Chem. 277 (2002) 30567–30573.
[24] D.A. Pye, R.R. Vives, P. Hyde, J.T. Gallagher, Regulation of FGF-1 mitogenic activity by heparan sulfate oligosaccharides is dependent on specific structural features: differential requirements for the modulation of FGF-1 and FGF-2, Glycobiology 10 (2000) 1183–1192.
[25] D.A. Pye, R.R. Vives, J.E. Turnbull, P. Hyde, J.T. Gallagher, Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity, J. Biol. Chem. 273 (1998) 2936–22942.
[26] C. Richard, M. Roghani, D. Moscatelli, Fibroblast growth factor (FGF)-2 mediates cell attachment through interactions with two FGF receptor-1 isoforms and extracellular matrix or cell-associated heparan sulfate proteoglycans, Biochem. Biophys. Res. Commun. 276 (2000) 399–405.
[27] J.E. Turnbull, D.G. Fernig, Y. Ke, M.C. Wilkinson, J.T. Gallagher, Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate, J. Biol. Chem. 267 (1992) 10337–10341.
[28] A. Walker, J.E. Turnbull, J.T. Gallagher, Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor, J. Biol. Chem. 269 (1994) 931–935.
[29] S. Ye, Y. Luo, W. Lu, R.B. Jones, R.J. Linhardt, I. Capila, T. Toida, M. Kan, H. Pelletier, W.L. McKeehan, Structural basis for interaction of FGF-1, FGF-2, and FGF-7 with different heparan sulfate motifs, Biochemistry 40 (2001) 14429–14439.
[30] S. Faham, R.E. Hileman, J.R. Fromm, R.J. Linhardt, D.C. Rees, Heparin structure and interactions with basic fibroblast growth factor, Science 271 (1996) 1116–1120.
[31] M.A. Nugent, R.V. Iozzo, Fibroblast growth factor-2, IJBCB 32 (2000) 115–120.
[32] A. Bikfalvi, S. Klein, G. Pintucci, D.B. Rifkin, Biological roles of fibroblast growth factor-2, Endocr. Rev. 18 (1997) 26–45.
[33] H.E. Conrad, Heparin-Binding Proteins, Academic Press, San Diego, 1998.

[34] C.J. Dowd, C.L. Cooney, M.A. Nugent, Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding, J. Biol. Chem. 274 (1999) 5236–5244.
[35] F.J. Moy, A.P. Seddon, P. Bohlen, R. Powers, High-resolution solution structure of basic fibroblast growth factor determined by multidimensional heteronuclear magnetic resonance spectroscopy, Biochemistry 35 (1996) 13552–13561.
[36] J.A. Abraham, J.L. Whang, A. Tumolo, A. Mergia, J. Friedman, D. Gospodarowicz, J.C. Fiddes, Human basic fibroblast growth factor: nucleotide sequence and genomic organization, EMBO J. 5 (1986) 2523–2528.
[37] Y. Duraisamy, J. Gaffney, M. Slevin, C.A. Smith, K. Williamson, N. Ahmed, Aminosalicylic acid reduces the antiproliferative effect of hyperglycaemia, advanced glycation endproducts and glycated basic fibroblast growth factor in cultured bovine aortic endothelial cells: comparison with aminoguanidine, Mol. Cell. Biochem. 246 (2003) 143–153.
[38] Y. Duraisamy, M. Slevin, N. Smith, J. Bailey, J. Zweit, C. Smith, N. Ahmed, J. Gaffney, Effect of glycation on basic fibroblast growth factor induced angiogenesis and activation of associated signal transduction pathways in vascular endothelial cells: possible relevance to wound healing in diabetes, Angiogenesis 4 (2001) 277–288.
[39] F. Facchiano, A. Lentini, V. Fogliano, S. Mancarella, C. Rossi, A. Facchiano, M.C. Capogrossi, Sugar-induced modification of fibroblast growth factor 2 reduces its angiogenic activity in vivo, Am. J. Pathol. 161 (2002) 531–541.
[40] I. Giardino, D. Edelstein, M. Brownlee, Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity. A model for intracellular glycosylation in diabetes, J. Clin. Invest. 94 (1994) 110–117.
[41] N.N. Nissen, R. Shankar, R.L. Gamelli, A. Singh, L.A. DiPietro, Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation, Biochem. J. 338 (1999) 637–642.
[42] G. Pintucci, N. Quarto, D.B. Rifkin, Methylation of high molecular weight fibroblast growth factor-2 determines post-translational increases in molecular weight and affects its intracellular distribution, Mol. Biol. Cell 7 (1996) 1249–1258.