A novel role for nitric oxide in the endogenous degradation of heparan sulfate during recycling of glypican-1 in vascular endothelial cells

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We show here that the endothelial cell-line ECV 304 expresses the heparan sulfate proteoglycan glypican-1. The predominant cellular glycoform carries truncated side-chains and is accompanied by heparan sulfate oligosaccharides. Treatment with brefeldin A results in accumulation of a glypican proteoglycan with full-size side-chains while the oligosaccharides disappear. During chase the glypican proteoglycan is converted to partially degraded heparan sulfate chains and chain-truncated proteoglycan, both of which can be captured by treatment with suramin. The heparan sulfate chains in the intact proteoglycan can be depolymerized by nitrite-dependent cleavage at internally located N-unsubstituted glucosamine moieties. Inhibition of NO-synthase or nitrite-deprivation prevents regeneration of intact proteoglycan from truncated precursors as well as formation of oligosaccharides. In nitrite-deprived cells, formation of glypican proteoglycan is restored when NO-donor is supplied. We propose that, in recycling glypican-1, heparan sulfate chains are cleaved at or near glucosamines with unsubstituted amino groups. NO-derived nitrite is then required for the removal of short, nonreducing terminal saccharides containing these N-unsubstituted glucosamine residues from the core protein stubs, facilitating re-synthesis of heparan sulfate chains.

Key words: brefeldin A/glypican/heparan sulfate/nitric oxide/suramin

Introduction

Cell-surface proteoglycans (PGs) are integrated in the plasma membrane via a hydrophobic region of the core protein (e.g., members of the syndecan family and betaglycan), or via a glycosylphosphatidylinositol (GPI)-anchor as in the glypican family (for reviews, see David, 1993; Bernfield et al., 1999) as well as in an isoform of brevican (Seidenbecher et al., 1995). Glypicans are exclusively substituted with heparan sulfate (HS); the syndecans and betaglycan may also contain chondroitin sulfate (CS), whereas brevican contains only CS. PG core proteins are synthesized on ribosomes attached to the endoplasmic reticulum (ER) and are translocated into the ER lumen. Glycosaminoglycan (GAG)-chain initiation starts at the serine of certain consensus sequences, e.g., D/EGSGD/E, by formation of the common linkage region GlcUA-Gal-Gal-Xyl-Ser. The HS chain is elongated on the linkage region by alternating addition of GlcNAc and GlcUA. Concomitantly, or lagging slightly behind, the chain is modified in different ways. Some of the GlcNAc moieties are N-deacetylated to GlcNH2 and, in most cases, immediately N-sulfated yielding GlcNSO3. GlcUA adjacent to GlcNSO3 is C-5 epimerized to IdoUA which, in turn, can be 2-O-sulfated. In addition, GlcNSO3 can be 6-O-sulfated and rare modifications include 3-O-sulfated GlcNSO3 and 2-O-sulfated GlcUA (for review, see Lindahl et al., 1998). It is generally assumed that most of the glycosyltransferases, polymerases, sulfotransferases, and modifying enzymes catalyzing these processes reside in the trans-Golgi complex. Heparan glucosaminyl N-deacetylation/N-sulfotransferase has indeed been located to this compartment (Humphries et al., 1997).

When synthesis is completed, PGs are guided by their core protein to different sites, such as intracellular storage vesicles, the extracellular matrix, or the cell surface. Glypicans, which acquire their GPI-anchor in the ER, are found at the surface of many cell types. The exact function of the lipid-anchor is not clear. In polarized cells, it may serve to direct glypican to the apical surface. Many GPI-anchored proteins are concentrated to caveolae, specific plasmalemmal structures believed to be involved in endo-/transcytosis and signaling (for review, see Anderson, 1998). Cell-surface associated HS-containing PGs (HSPG) support a wide range of biological functions, including growth control, cell adhesion, endo- and transcytosis and anticoagulant activity (for references, see Mertens et al., 1992; Misra et al., 1994; Taipale and Keski-Oja, 1997; Lindahl et al., 1998; Bernfield et al., 1999).

Normal human vascular endothelial cells produce a variety of membrane-bound HSPGs, mainly syndecans but also glypican-1 (Mertens et al., 1992). It has also been reported that endothelial PGs carry HS-chains with N-unsubstituted glucosamines (GlcNH2) which serve as recognition and binding sites for L-selectin (Norgard-Sumnicht and Varki, 1995). By using a specific monoclonal antibody that recognizes these GlcNH2 residues, van den Born et al. (1995) detected an uneven distribution of this epitope among renal basement membranes.

The metabolic turnover of membrane-bound HSPGs follows different routes. Transmembrane intercalated forms are either internalized by endocytosis and degraded stepwise in endo-
somnes or lysosomes, or cleaved proteolytically and shed into the extracellular space (Yanagashita and Hascall, 1992; Bernfield et al., 1999). Glypicans can, in addition, be cleaved at the phosphate-inositol bond by phosphatidylinositol-specific phospholipase C resulting in release of the PG from its lipid-anchor (Schmidtchen et al., 1990; David, 1993). Recycling of a phospholipase C-resistant variant of fibroblast glypic an has been proposed (Fransson et al., 1995). This proposal was based on the observation that a HSPG with the properties of glypic an was still being HS-chain radiolabeled when cells were treated with brefeldin A (BFA), an inhibitor of transport of newly-made core protein from the ER to the Golgi (Klausner et al., 1992, and references therein). Furthermore, cell-surface HSPG tagged with biotin reappeared radiolabeled after incubation of cells with $^{35}$S sulfate in the presence of BFA. It was suggested that the glypic an variant recycles via endosomes to the trans-Golgi compartment. During recycling HS-chains were partially degraded and resynthesized on the remaining stubs, and the reprocessed PG was then returned to the cell surface (Edgren et al., 1997). Suramin, which inhibits both internalization and degradation of HS (Nakajima et al., 1991; Voogd et al., 1993, and references therein), resulted in accumulation of glypican-like HSPG (Fransson et al., 1995).

Results obtained in previous studies on PGs synthesized by human umbilical vein endothelial cells suggested a non-enzymatic, autodegradation of HS in cell extracts (Lindblom et al., 1989; Lindblom; Fransson, et al., 1998). Vilar et al. (1997) showed that endothelial cells can generate sufficient amounts of NO (and subsequently nitrite) to support degradation of exogenously added HS. We have now used a vascular endothelial cell-line (ECV 304) and the drugs BFA and suramin to arrest endogenously formed HSPGs at various stages of their turnover and recycling. Our results indicate that simultaneous manipulations of NO-formation or nitrite-deprivation affects the degradation of HS and the recycling of glypican-1.

Results
Effects of BFA and suramin on proteoglycan production
BFA is known to inhibit transport from the ER to the Golgi in all cells (Klausner et al., 1992). In polarized cells, such as endothelial cells, there could also be effects of BFA on vesicular trafficking to and from apical membrane domains as endosomes involved in recycling contain BFA-sensitive coat proteins (Hunziker et al., 1991; Smart et al., 1994). Suramin is known to inhibit both internalization and enzymatic degradation of HS (Nakajima et al., 1991). To test the effects of these drugs on PG-metabolism, endothelial cells (ECV 304) were metabolically labeled with $^{35}$S sulfate in the absence or presence of BFA or suramin or both. Polyanionic macromolecules were isolated from the Triton X-100 extract of the cells either by Alcian blue precipitation followed by SDS–PAGE (Figure 1A–C) or by recovery on DEAE-cellulose followed by gel-exclusion chromatography on Superose 6 (Figure 1D–F, solid line). The radiolabeled material from untreated cells consisted of two pools: material that migrated with an apparent Mr of 100–200 kDa on SDS–PAGE (Figure 1A) and eluted as a broad peak on Superose 6 (see bar in Figure 1D) and smaller-size material of oligosaccharide-type (Mr < 50 kDa in Figure 1A) that was well retarded on Superose 6 (Figure 1D). The larger material was sensitive both to digestion with HS lyase (Figure 1A) and to alkali-treatment (Figure 1D, dashed line) indicating that it consisted of HS linked to core protein. Also the pool of smaller-size oligosaccharide-like material was degraded by HS lyase (Figure 1A). The yields of HSPG and HS-oligosaccharide were the same after extraction with 4 M guanidine/Triton X-100 and after addition of heparin (1 mg/ml) to the Triton X-100 extractant. Pre- and postconfluent cells also afforded similar proportions of the two materials (data not shown).

In contrast, BFA-treated cells accumulated large, cell-associated PGs (Mr 100–250 kDa or higher) whereas very little small-size products could be detected (Figure 1B,F). The GAG chains in the PG-pool were completely degraded by digestion with HS lyase (Figure 1B). Results of alkali treatment (Figure 1E, dashed line) indicated that this PG pool contained larger HS-chains (Kav 0.24) than the material from untreated cells (Kav 0.5–0.7). In the medium of BFA-treated cells no PG could be detected (data not shown).

In suramin-treated cells, intermediate-size, HS-lyase sensitive products accumulated (Figure 1C,F). One portion of the material was sensitive to alkali-treatment, whereas another was not (Figure 1F, dashed line) indicating that the material consisted of both free HS-fragments and HS attached to protein. The average size of the suramin-arrested HS-material (protein-bound as well as unbound) was somewhat larger (Kav 0.7) than that of the HS-oligosaccharides (Kav 0.9) isolated from untreated cells (Figure 1D). When BFA and suramin were combined, the result was the same as with BFA alone (data not shown). Hence, the BFA-block should precede that of suramin.
Nitric oxide and heparan sulfate degradation

Glypican-1 proteoglycan accumulates in BFA-treated cells

The nature of the BFA-arrested HSPGs was determined by immunodetection after electrophoresis of the core proteins and by direct immunoisolation from the cell extracts. To be able to trace the material during purification, PGs were metabolically labeled with [35S]sulfate. Cell-associated PG was extracted from the cell layer, recovered by passage over DEAE-cellulose, and purified by gel permeation chromatography on Superose 6 (as in Figure 1E) followed by ion-exchange chromatography on Mono Q (Figure 2A). PG material (see bar) was pooled and digested with HS lyase followed by SDS–PAGE. Western blot using immunostaining with monoclonal antibodies S1 or 2E9 specific for glypican-1 and syndecan-1/3, respectively, was then performed (David et al., 1990; Mertens et al., 1992). The result (see inset in Figure 2 A) showed one core protein (66 kDa) reacting with glypican-1 antibody and another one (125 kDa), which was either that of syndecan-3 or aggregated syndecan-1 core protein (Bernfield et al., 1999).

Radiolabeled, cell-associated glypican-1 was also directly immunoisolated using a polyclonal anti-glypican-1 antiserum and subjected to SDS–PAGE (Figure 2B). In untreated cell cultures, a relatively small, [35S]sulfate-labeled glypican-1 glycoform (M, approx. 100–200 kDa) was obtained from the cell extract, whereas smaller amounts of a larger form were recovered from the medium. In BFA-treated cells a large, but polydisperse glycoform of glypican-1 (M, approx. 100–300 kDa or higher) accumulated. A separate minor component of M, approx. 90 kDa was also seen.

The BFA-arrested material is a precursor of the suramin-arrested material

Pulse-labeling of BFA-treated cells with [35S]sulfate followed by extraction, precipitation with Alcian blue and SDS–PAGE indicated that the accumulation of PG increased up to 12 h and then remained relatively constant (data not shown). In a pulse-chase experiment, cells were incubated with [35S]sulfate for 12 h and then chased in either drug-free or suramin-containing nonradioactive medium. Cells were extracted and aliquots were chromatographed on Superose 6 (Figure 3). After 24 h of chase in drug-free medium, prelabeled PG material had been converted to intermediate-size products eluting in fractions 29–38 (Figure 3B, solid line) and after 42 h most of this had disappeared (Figure 3C, solid line). Throughout the chase, a minor portion of PG-material remained undegraded. Oligosaccharides, expected to elute in fractions 40–44, were not seen. No PG or oligosaccharide could be found in the medium (data not shown). However, during chase in suramin-containing medium, both intermediate-size products and oligosaccharides accumulated (Figure 3B,C, dashed lines). Hence, suramin can block further degradation of HS-material released from the BFA-block.

Detection of glucosamine residues with unsubstituted amino groups in heparan sulfate chains

The [35S]-labeled HSPG and HSPG-degradation products that accumulated in BFA- and suramin-treated cells, respectively, were isolated as described in Figure 1 and treated with alkali to release protein-bound chains and chain fragments. The samples were then chromatographed on Superose 6 before and after deaminative cleavage at pH 3.9 which is specific for

Fig. 2. Identification of glypican-1 PG forms. Cells were grown to confluence in (A) 175 cm² or (B) 75 cm² dishes and incubated in sulfate-deficient medium with 50 µCi/ml [35S]sulfate for (A) 24 h or (B) 16 h in the absence or presence of BFA (BFA, 10 µg/ml). The cell layers were washed, extracted with Triton X-100 and (in A) polyanionic material from BFA-treated cells was collected by passage over DEAE-cellulose, followed by gel-permeation chromatography on Superose 6 (as in Figure 1E). Macromolecular [35S]material was pooled and subjected to ion-exchange FPLC on Mono Q. Elution was performed with a linear gradient from 0.3 M NaCl to 1.2 M NaCl (dashed line). The elution positions of standard heparan sulfate (HS) and dermatan sulfate (DS) are indicated. The pooled PG material (see bar) was precipitated with ethanol, digested with HS-lyase and subjected to 10% SDS–PAGE under nonreducing conditions (see inset). After blotting to nylon membrane this was immunostained with (lane 1) monoclonal S1 antibody against glypican and (lane 2) monoclonal 2E9 antibody against syndecans 1 and 3. Molecular size markers are indicated. Exposure times were 10 sec in lane 1 and 10 min in lane 2. In (B) glypican-1 glycoforms were isolated from (M) media and (C) SDS-Triton-deoxycholate extracts of cells by immunoadsorption and subjected to 3–12 % SDS–PAGE under reducing conditions. Bands were visualized by autoradiography.
glucosamines with unsubstituted amino groups (Lindahl et al., 1973). It is seen (Figure 4A) that the large HS-chains derived from the BFA-arrested PG (solid line) were partially degraded by nitrous acid to yield HS-fragments (dashed line) of the same size as those accumulating in suramin-treated cells (Figure 4B, solid line). In contrast, the suramin-arrested chain fragments were not extensively depolymerized by nitrous acid at pH 3.9 (Figure 4B, dashed line). Hence, cleavage at or near the GlcNH₂ units of the HS-chains in the BFA-arrested PG seems to take place when the suramin-arrested material is formed.

Although suramin-arrested HS-degradation products were not depolymerized by nitrous acid, this does not exclude the possibility that GlcNH₂ units were present in, e.g., non-reducing terminal position. As endogenously produced nitrite

**Fig. 3.** Conversion of BFA-arrested proteoglycan to degradation products during chase. Cells grown to confluence in 25 cm² dishes were (A) incubated with [³⁵S]sulfate in the presence of BFA (10 μg/ml) for 12 h followed by chase in non-radioactive medium for (B) 24 h and (C) 42 h (solid line) without further additions and (dashed line) with the addition of suramin (0.2 mM). The media were removed and the cell layers were extracted with Triton X-100. Aliquots (200 μl) of the extracts were mixed with 8 M guanidine HCl (1:1) and chromatographed on Superose 6.

**Fig. 4.** Depolymerization at N-unsubstituted glucosamine in heparan sulfate chains. Gel chromatography on Superose 6 of HS-chains/fragments derived via alkaline elimination from (A) the BFA-arrested PG and (B) the suramin-arrested material. The products were analyzed (solid line) without further treatment and (dashed line) after treatment with HNO₂ at pH 3.9. Confluent cells (25 cm² dishes) were incubated in sulfate-deficient medium containing 50 μCi/ml [³⁵S]sulfate in the presence of BFA (10 μg/ml) or suramin (0.2 mM) at 37°C for 24 h. The medium was removed and cells were washed and extracted with Triton X-100. PG and PG-derived material of the cell extract were recovered by passage over DEAE-cellulose, treated with alkali to release chains from the core protein and again recovered on DEAE-cellulose.
produced by decomposition of arginine catalyzed by NO-

Nitrite is formed by oxidation of NO which, in turn, is produced by decomposition of arginine catalyzed by NO-

Inhibitors of nitrite and nitrite-generation affect proteoglycan and oligosaccharide production

Nitrites, the constitutive form cNOS and the inducible form NO-donor (for review, see Wink et al., 1996). NO is unstable and either directly converted to nitrite or stored as protein-bound S-nitrosothiols. The latter release NO non-enzymatically in a process catalyzed by Cu²⁺ (for review, see Williams, 1996). To explore whether NO-derived nitrite was involved in HS-degradation, cells were pre-exposed to the NO-synthase inhibitors N-methyl-arginine (inhibits both forms of NOS), N-nitroarginine (inhibits preferentially cNOS) or aminoguanidine (inhibits preferentially iNOS) or to the nitrite-degrading agent ammonium sulfamate. Cells were then incubated with radiosulfate in the low-arginine medium 199 in the continued presence of the respective compounds. As shown in Figure 5A–E treatment with these compounds reduced generation of HS-oligosaccharides (fractions 40–45). The order of effectiveness was N-methylarginine = sulfamate > aminoguanidine > N-nitroarginine. There was, however, no concomitant increase in the radiolabeling of HSPG (fractions 20–25), as was seen when cells not deprived of nitrite were treated with BFA (Figure 5F).

When cells had been pre-treated with suramin, which would result in accumulation of intermediate-size products (see Figure 1F), and then radiosulfated in the presence of BFA accumulation of large HSPG was resumed (Figure 5G) suggesting that the suramin-block also precedes the BFA-block in a cyclic process. Moreover, formation of large HSPG under these conditions (Figure 5G) was marked reduced by the simultaneous presence of sulfamate (Figure 5H) suggesting that a nitrite-dependent step could be located downstream of the suramin-block but upstream of the BFA-block. This was further supported by the finding that formation of radiolabeled HSPG in BFA-treated cells was inhibited by pre-incubation with aminoguanidine (Figure 5I) or sulfamate (Figure 5J).

To test whether nitrite was also involved in the degradation of HS-chain fragments and oligosaccharides, radiosulfate-labeled suramin-arrested material was chased in nonradioactive medium with or without sulfamate and the products were analyzed as in Figure 3. The turnover of HS-degradation products was unaffected by the presence of sulfamate (data not shown). However, as shown above, supply of nitrite appears necessary to sustain formation of the BFA-arrested HSPG, perhaps by facilitating HS-chain extension. Hence, we tested whether HS-chain extension and formation of the BFA-arrested HSPG could be restored in nitrite-deprived cells when NO-donor was supplied.

Formation of BFA-arrested proteoglycan in nitrite-deprived cells is restored by NO-donor

Cells were deprived of nitrite by treatment with a combination of aminoguanidine (to inhibit iNOS), sulfamate (to destroy nitrite), and neocuproine (to inhibit release of NO from S-nitrosothiols). The cells were then chased in fresh, BFA-containing medium with [35S]sulfate and increasing concentrations of NO-donor (sodium nitroprusside). [35S]PG was isolated from the cell extracts by ion-exchange chromatography. As seen in Figure 6A (solid circles) formation of BFA-arrested HSPG was gradually stimulated in the presence of increasing concentrations of NO-donor. Maximal stimulation occurred at 300 µM nitroprusside or higher. The stimulated PG-formation reached the same level as in cells not deprived of nitrite and not treated with NO-donor (open circles).
To determine which type(s) of HSPG that is formed during NO-stimulation, it was necessary to generate both protein- and carbohydrate-labeled, BFA-arrested HSPG. Pilot experiments indicated that radiolabeling of untreated cells with \[^{35}S\]methionine generated immunoreactive and radiolabeled truncated glypican-1, but radiolabeling was greatly reduced in
the BFA-arrested glypican-1 (as in Figure 2B) and insufficient for core protein analysis (data not shown). Using [3H]leucine, suramin-arrested radiolabeled material (purified on MonoQ) could be detected (data not shown). Although an accumulation of this radiolabel could be seen in large HSPG after chase in nonradioactive medium containing BFA, the amounts were again insufficient for core protein analysis (data not shown). We therefore tested whether sufficient amounts of radiolabeled core protein precursor could be obtained by arresting material at a nitrite-dependent step.

Subconfluent cell cultures were metabolically labeled with [35S]methionine/cysteine until cells were confluent (usually within 24 h) and then radiolabeling was continued for another 24 h in the presence of aminoxyanidine, sulfamate, and neocuproin to minimize the endogenous nitrite concentration. Cells were then chase-labeled overnight in fresh BFA-containing medium with [3H]glucosamine (to label the HS backbone) and in the absence or presence of 300 µM sodium nitroprusside as NO-donor. Radiolabeled PG was recovered from the cell extracts by passage over DEAE-cellulose and then chromatographed on Superose 6 (Figure 6B). It is seen that very little [3H]PG was formed in unstimulated cells (dashed line), whereas a large [3H]PG peak was obtained from stimulated cells (solid line).

To obtain HSPG that could be used for core protein analysis, further purification by ion exchange chromatography on MonoQ was necessary in order to remove some non-PG protein (Figure 6C,D). The [35S]methionine/cysteine-labeled PG recovered from (C) pulse-labeled and (D) chase-labeled cells were thus isolated (see bars in Figure 6C,D). HS-chains were removed by digestion with HS lyase and [35S]core proteins were subjected to SDS–PAGE (see inserts in Figure 6C,D). After the pulse, a HSPG with a core protein of approx. 120 kDa was seen (Figure 6C). After the chase in medium containing BFA and NO-donor, an additional HSPG with a core protein of ~70 kDa appeared (Figure 6D). This size corresponds to that of glypican (David et al., 1990; Schmidtchen et al., 1990).

Discussion

Our results show that a transformed endothelial cell-line derived from human umbilical vein endothelial cells (ECV 304) express glypican-1. In unperturbed cells glypican-1 is mostly in the form of HS-chain truncated glycoforms accompanied by relatively large amounts of HS oligosaccharides (Figure 7). Very little large glypican PG is found associated with the cell layer but some is shed into the medium. In BFA-treated cells, large glypican PG accumulates and shedding ceases. Radiolabeling of the core protein in the BFA-arrested PG was much reduced, as if there is an available pool of nonradioactive precursors. These could be HS-chain truncated glycoforms in various stages of degradation or reconstruction as depicted in Figure 7. According to the proposed recycling model, formation of HS-oligosaccharides should cease when glypican-1 is arrested in the large PG form. The HS-oligosaccharide pool then becomes depleted by terminal degradation in lysosomes.

From the recycling model further predictions can be made. The large glypican PG should be the precursor of the suramin-arrested truncated form and recycling back to the BFA-arrested form should be precluded by nitrite-deprivation. Accordingly, during chase the BFA-arrested glypican-1 PG was degraded by endoglycosidic cleavage, presumably in the vicinity of the GlcNH₂ moieties, generating HS-chain fragments and oligosaccharides. Furthermore, during chase in the presence of suramin both truncated PG and HS fragments could be captured. By arresting recycling material at the suramin stage and then chasing in the presence of BFA the large glypican PG was reformed. This route was also abrogated by nitrite-deprivation as was subsequent generation of HS-oligosaccharides. Core protein analysis revealed that glypican PG was reformed from prelabeled material in nitrite-deprived cells upon addition of NO-donor. During recycling no intermediates were seen in the medium, suggesting that it was an intracellular process.

Degradation of HS-side chains could take place in several stages (Figure 7). We propose an initial partial endoglycosidic degradation on the nonreducing side of the GlcNH₂ moieties of HS, releasing relatively large HS-chain fragments, and gradually generating a truncated PG with the GlcNH₂ moieties near the nonreducing end of the stubs. Endoglycosidases (heparanase) capable of partially depolymerizing HS chains of HSPG have been demonstrated in many cell types, including endothelial cells (Goddé et al., 1991). In most cases, these endoheparanases cleave β-D-glucuronic linkages which would generate truncated HSPG with stubs containing nonreducing terminal glucosamine residues (for an extensive list of references, see Sandbäck-Pikas et al., 1998). We have previously observed that HS oligosaccharides generated by endoglycosidic cleavage in fibroblasts had been cleaved at sites located close to the heparanase I cleavage sites, i.e., at the nonreducing side of IdoUA(2-OSO₃) (Schmidtchen and Fransson, 1994). Recent studies confirm this observation. CHO cell- (Bai et al., 1997) or human hepatoma- and platelet-derived endoheparanases (Sandbäck-Pikas et al., 1998), which are not specific for...
the type of N-substitution of the adjacent glucosamine, have a requirement for 2-O-sulfate on neighboring hexuronic acid residues located on the reducing side of the cleavage site (–) in, e.g., the following sequence: -GlcNH2-GlcUA-GlcNH2-HexUA(2-OSO3)-GlcNR- where R is an unspecified substituent and HexUA could be IdUA. Cleavage of a glucuronidic linkage a short distance from the non-reducing side of a GlcNH2 would thus generate the non-reducing terminal sequence GlcNR-HexUA(2-OSO3)-[GlcNR-HexUA]n-GlcNH2 in the core protein stubs (Figure 7). When n is small or zero subsequent deaminative cleavage at the reducing side of GlcNH2 would result in an undetectable/marginal effect on overall chain/stub size. It is possible that these short non-reducing terminal GlcNH2-containing “telosaccharides” are cleaved off when sufficient high concentrations of nitrite have been generated endogenously in a mildly acidic compartment providing fresh acceptor sites for HS chain extension (Figure 7). The “telosaccharides” may contain unexpected features, such as 3-O-sulfation. It is intriguing that certain isoforms of 3-O-sulfatase (e.g. 3-OST-3) recognize IdUA(2-OSO3)-GlcNH2 repeats (Shukla et al., 1999). It should be added that in fibroblasts, there may be no need for NO-derived nitrite as fibroblast HS chains do not appear to contain much GlcNH2 (Fransson et al., 1995).

The intracellular location of the proposed events has not been specifically addressed. However, NO, the precursor of nitrite and thus a potential regulator of glypican recycling, may be formed both in caveolae and in endosomes. Constitutive endothelial NO-synthase (cNOS/eNOS) targets to the cytoplasmic side of caveolae when made lipophilic by acylation (Garcia-Cardenas et al., 1996; Shaul et al., 1996). However, NOS appears to be less active when it is associated with caveolin, the structural scaffolding protein of caveolae (Feron et al., 1996; Shaul et al., 1996). Therefore, effects on HS turnover may be seen when the amount of NO has reached a threshold level (e.g., >300 µM) in the local subcellular environment. This may occur when recycling glypicans passes through the endosomal compartment where NOS should be fully active. In this way NO could regulate the level and structure of cell-surface HS. Specific structures in glypican HS-chains contribute to the anticoagulant potency of the intact vascular endothelium by binding/activation of antithrombin III (Mertens et al., 1992). It is thus interesting that prolonged inhibition of NO-synthase decreases the expression of anticoagulant HS on endothelial cells (Irokawa et al., 1997). Recycling glypicans may also be involved in internalization and recycling of HTA-binding growth factors and enzymes (reviewed in Bernfield et al., 1999) or polyamines (Belting et al., 1999).

Materials and methods
The endothelial cell line ECV 304 was obtained from Prof. Inge Olsson, Department of Medicine, Lund University. Regular cell culture media, L-glutamine, penicillin-streptomycin, trypsin, and fetal bovine serum were obtained from Life Technologies. Heparin and other GAG were the same preparations as described earlier (Fransson et al., 1992). BFA, N02-methyl-L-arginine, Nnitro-L-arginine, aminoquinidine, neocuproine (2,3-dimethyl-1,10-phenanthroline), and medium 199 were purchased from Sigma, suramin (Germanine) from Bayer, and sodium nitroprusside from Fluka. Na235SO4 (1310 Ci/mmol), D-[6-3H]glucosamine (40 Ci/mmol), L-[35S]-methionine/L-[35S]-cysteine, 7/3 (>1000 Ci/mmol, Pro-Mix), PVDF-membranes, and Hybond N were from Amersham International, UK. HS lyase (heparitinase) was purchased from the Seikagaku Corporation, Japan. The packed columns (Superose 6 HR 10/30 and Mono Q HR 5/5), protein A–Sepharose CL–4B and Dextran T-500 was from Pharmacia-LKB, Sweden, DE-53 DEAE-cellulose from Whatman and Bio-Gel P-10 and protein standards from Bio-Rad. Centricon 30 was purchased from Amicon UK and Alcian blue 8GS from Chroma-Gesellschaft, Germany. A rabbit antiserum against glypican-1 was obtained after immunization with a 6-His tagged recombinant glypican-1 core protein comprising the sequence Ile 54 to Pro 519. To generate the protein human glypican-1 cDNA was cleaved with BglII and Stnl and ligated into the vector pQE32 (Qiagen) digested with BamHI/Smal. The resulting plasmid was used to transform E.coli M15 bacteria. Protein expression was induced with IPTG (Gibco BRC). 6-His tagged protein was purified in guanidineHCl on a Ni2+-NTA-agarose column (Qiagen). The antibodies Mab S1 and Mab 2E9 were kindly provided by Prof. Guido David, University of Leuven, Belgium. Chemiluminiscent Super signal substrate was from Pierce. Other chemicals were of analytical grade.

Cell culture and radiolabeling
ECV 304 (a transformed vascular endothelial cell line) was cultured as monolayers in Dulbecco’s Modified Earle’s Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) in an incubator with humidified atmosphere and 5% CO2 at 37°C. Confluent cells were preincubated for 1 h in labeling medium supplemented with 2 mM glutamine and serum. The labeling medium was low-sulfate (0.05 mM) MgCl2-containing DMEM with serum. When NO-synthase inhibitors/nitrite-quenchers were tested, cells were incubated in a low-arginine medium (M 199). Drugs used were: BFA (10 µg/ml) or suramin (0.2 mM). Radioactivity was measured by β-scintillation.

Extraction and isolation of cell-associated proteoglycans
After the incubations, medium was collected and pooled with two washings with ice-cold PBS (0.137 M NaCl, 3 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.5). Cells were extracted with 0.1–0.2 ml/cm2 dish of 0.15 M NaCl, 10 mM EDTA, 2% (v/v) Triton X-100, 10 mM KH2PO4, pH 7.5, 5 µg/ml ovalbumin containing 10 mM N-ethylmaleimide, and 1 mM diisopropylphosphoro-fluoridate on a slow shaker at 4°C for 10 min. Isolation and purification of PG was performed according to two different methods. Procedure I included chromatography on DEAE-cellulose, Superose 6, and Mono Q, and procedure II consisted of Alcian Blue precipitation (Björnsson, 1993) followed by electrophoresis (SDS–PAGE).

In procedure I extracts were mixed with 1.3 vol. of 7 M urea, 10 mM Tris, pH 7.5, 0.1% Triton X-100, 10 mM NEM and
passed over a 1 ml column of DE-53 equilibrated with 6 M urea, 0.5 M NaOAc, pH 5.8, 5 µg/ml ovalbumin, 0.1% Triton X-100. After sample application, the columns were washed successively with 10 ml portions of (1) equilibration buffer (see above); (2) 6 M urea, 10 mM Tris, pH 8.0, 5 µg/ml ovalbumin, 0.2% Triton X-100; and (3) 50 mM Tris pH 7.5. Bound material was eluted with 5 x 1 ml 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8, 5 µg/ml ovalbumin, 0.2% Triton X-100. Radioactive fractions were pooled, precipitated with 5 vol of 95% ethanol and 100 µg of dextran as carrier, overnight at –20°C. Samples were centrifuged in a Beckman JS-7.5 at 4000 r.p.m. and 4°C for 45 min and dissolved in 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8, 0.2% Triton X-100. They were then subjected to gel permeation FPLC on Superose 6 at a flow rate of 0.4 ml/min in the same buffer. Radioactivity was activity and degradation steps. Samples were chromatographed on Mono Q after buffer-change on Centricon 30. The buffer used was 7 M urea, 10 mM Tris, pH 8.0, 0.1% Triton X-100 and the gradient was between 0.3 M NaCl (fraction 10) to 1.2 M NaCl (fraction 70) in the same buffer. Radioactive PGs were pooled and precipitated as above.

Immunosolisation

Cell medium was decanted, and the cell layer was washed with PBS and solubilized in PBS containing 0.1% (w/v) SDS, 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate by passage up and down a glass pipette at 4°C for 10 min. After addition of PMSF (a 1/1000 dilution of a saturated solution in ethanol), the medium and cell extract were treated with protein A Sepharose CL-4B (1/100) for at least 1 h on a slow shaker at 4°C. The supernatant was collected and treated with anti-glypican-1 antiserum (diluted 1/200) at 4°C for 1 h, then further incubated for 1 h with horseradish peroxidase–conjugated anti-mouse IgG diluted 1:5000 in washing buffer. After three washings in washing buffer and two washings in TBS (100 mM Tris, 0.9% NaCl, pH 7.5), the membrane was finally developed with Supersignal substrate for chemiluminiscence and autoradiographed.

Degradation procedures

GAG chains were released from PG by treatment with 0.5 M NaOH, 0.1 M NaBH₄ at room temperature overnight. Samples were neutralized with HOAc, freeze-dried, and redissolved for analysis by gel-permeation chromatography on Superose 6 as above. Cleavage of N-unsubstituted glucosamines was carried out with nitrous acid at pH 3.9 (Lindahl et al., 1973). HS chains were also degraded with HS lyase (3 mU/ml) in the presence of proteinase inhibitors as described (Fransson et al., 1995). Digestions were terminated by heating at 100°C for 1 min. The volume of samples was reduced and buffer changes were made by centrifugation in Centricon 30. Material was recovered by ethanol precipitation, dissolved in SDS-buffer and analyzed on SDS-PAGE. Carrier protein (ovalbumin) and HS or dextran (50–100 µg) were added prior to each purification and degradation step.

SDS-PAGE

Radiolabeled PG and HS lyase-treated PG were dissolved in SDS-buffer consisting of 5% (w/v) SDS, 20% (v/v) glycerol, 4 mM EDTA, 0.04% bromphenol blue, 125 mM Tris–HCl, pH 6.8, and 10% (v/v) β-mercaptoethanol. The samples were boiled for 2 min before loading. For immunostaining gels were equilibrated in transfer buffer (92 mM glycine, 0.01 M Tris, pH 8.3, 20% methanol) for 30 min. Transfer to PVDF-membrane was carried out overnight at 4°C and a constant voltage of 20 V. After a 1 h exposure to PBS containing 10% milk (3% fat), the membrane was incubated with 20 µg/ml Mab S1 (anti-glypican) or 20 µg/ml Mab ZE9 (anti-syndecan 1+3) in PBS containing 5% milk (washing buffer) for 2 h. The membrane was rinsed twice for 5 min with washing buffer and further incubated for 1 h with horseradish peroxidase–conjugated anti-mouse IgG diluted 1:5000 in washing buffer. After three washings in washing buffer and two washings in TBS (100 mM Tris, 0.9% NaCl, pH 7.5), the membrane was finally developed with Supersignal substrate for chemiluminiscence and autoradiographed.

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Abbreviations

Brefeldin A, BFA; CS, chondroitin sulfate; ECV, endothelial cell-line of vascular origin; ER, endoplasmic reticulum; GAG, glycosaminoglycan; Gal, galactose; GlcNH₂, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; GlcNOS, N-sulfate-D-glucosamine; GlcNR, glucosamine with unspecified N-substituent; GlcUA, D-glucuronic acid; GPI, glycosylphosphatidylinositol; HexUA, unspecified hexuronic acid; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IdOUA, L-iduronic acid; NOS, nitric oxide synthase; PG, proteoglycan; -OSO₃, sulfate ester; Xyl, xylose.

References


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