Dermatan Sulfate Released after Injury Is a Potent Promoter of Fibroblast Growth Factor-2 Function*

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‡‡‡ The abbreviations used are: GAG, glycosaminoglycan; FGF, fibroblast growth factor; FGFR1, FGF receptor-1; BSA, bovine serum albumin; ACE, affinity co-electrophoresis; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; WFGAG, wound fluid GAG; mAb, monoclonal antibody.

Proteoglycans have been shown in vitro to bind multiple components of the cellular microenvironment that function during wound healing. To study the composition and function of these molecules when derived from an in vivo source, soluble proteoglycans released into human wound fluid were characterized and evaluated for influence on fibroblast growth factor-2 activity. Immunoblot analysis of wound fluid revealed the presence of syndecan-1, syndecan-4, glypican, decorin, perlecan, and versican. Sulfated glycosaminoglycan concentrations ranged from 15 to 65 µg/ml, and treatment with chondroitinase B showed that a large proportion of the glycosaminoglycan was dermatan sulfate. The total glycosaminoglycan mixture present in wound fluid supported the ability of fibroblast growth factor-2 to signal cell proliferation. Dermatan sulfate, and not heparan sulfate, was the major contributor to this activity, and dermatan sulfate bound FGF-2 with $K_a = 2.48 \mu M$. These data demonstrate that proteoglycans released during wound repair are functionally active and provide the first evidence that dermatan sulfate is a potent mediator of fibroblast growth factor-2 responsiveness.

Proteoglycans are glycosaminoglycan (GAG)$^1$-containing molecules characterized by core protein structure and the size and type of associated GAG(s) (1–4). Heparan sulfate GAGs bind a plethora of molecules including several growth factors, cytokines, cell adhesion molecules, matrix proteins, proteases, and antiproteases (2, 5–9). Although the biological significance of these binding interactions remains unclear, the unique ability to interact with a vast array of ligands enables proteoglycans to influence several cell behaviors. This influence becomes of particular importance during inflammation and the response to injury when heparan sulfate-containing proteoglycans are thought to control the interaction between numerous cells, matrix components, and soluble effectors (10, 11). Dermatan sulfate may also interact with several molecules primarily thought of as heparan sulfate-binding proteins, e.g. fibroblast growth factor-2 (FGF-2) (12), hepatocyte growth factor/scatter factor (13), heparan cofactor II (14, 15), platelet factor 4 (16), fibroactin (17), and protein C inhibitor (18). Thus, multiple types of GAG are likely important during inflammation, the response to injury, and related processes.

Proteoglycan expression is regulated during wound repair. Abundant syndecan-1 and -4 are induced in response to injury on endothelia and fibroblasts, cell types that normally express only low amounts of these proteoglycans (19, 20). Soluble syndecan-1 and -4 extracellular domains have also been identified in wounds (21). The presence of both soluble and cell-surface proteoglycans provides multiple sites where cell behaviors required for wound healing may be regulated. However, although proteoglycan function has been extensively studied in vitro, little is known about native proteoglycans or GAG released in vivo.

To determine whether soluble proteoglycan and/or GAG isolated from an in vivo source can function to support cell behaviors mediated by GAG-binding ligands, we studied the ability of purified human wound fluid GAG (WFGAG) to support FGF-2-mediated cell proliferation. Fibroblast growth factors (FGFs) are an important family of at least nine structurally related GAG-binding molecules (11). FGF-2, the best characterized member of the FGF family, is present in wounds and can function as a mitogen that signals mesenchymal cell migration, proliferation, and differentiation (22–25). The interaction between FGF-2 and heparin, or heparan sulfate, has been well characterized, and function has been reported to depend on binding to these GAGs (26–28). A model has been proposed in which cell-surface heparan sulfate functions as a low affinity co-receptor for FGF-2 and presents the growth factor to its high affinity signaling receptors (27–29).

We report the presence in wounds of abundant soluble GAG, largely in the form of dermatan sulfate, that supports the ability of FGF-2 to signal cell proliferation. Elimination of dermatan sulfate from WFGAG resulted in an 85% reduction in FGF-2 activity. These findings directly demonstrate that proteoglycans released from in vivo sources can support cell behaviors required for wound healing. Furthermore, this study demonstrates that in addition to the ability of heparan sulfate to support FGF-2 activity, soluble dermatan sulfate also supports FGF-2-mediated cell proliferation and is likely an important molecule involved in the regulation of wound repair.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FGF-2 was the generous gift of Dr. M. Klagesbrun, Harvard Medical School (Boston). Bovine serum albumin (BSA), chondroitin sulfate B lyase (chondroitinase B), porcine intestinal heparin, chondroitin sulfate ABC, and heparan sulfate were from Sigma. Unless indicated, porcine skin chondroitin sulfate B (dermatan sulfate) with molecular mass range 11–25 kDa and determined pure by infrared spectrophotometry was from Seikagaku America Inc. (Rockville, MD). The total nitrogen, sulfur, galactosamine, and iduronic acid

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content of this dermatan sulfate preparation was 2.82, 6.77, 32.9, and 39.0%, respectively. The Blyscan Proteoglycan and GAG Assay System was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Complete EDTA-free Proteases Inhibitor Mixture was from Boehringer Mannheim. IODO-BEADS were purchased from Porvair Sciences. Monoclonal antibody DL-101 specific for human syndecan-1 ectodomain was kindly provided by Dr. M. Bernfield, Harvard Medical School (Boston). Monoclonal antibodies 10H4 (30) and IC3 (31) specific for human syndecan-2 and -3 ectodomains, respectively, and monoclonal antibodies against human perlecan (matrix mix) (32) and glypican (SI) (33) were generously provided by Dr. G. David, University of Leuven (Leuven, Belgium); proteoglycan cells were kindly provided by Dr. R. Howard, University of Leuven (Leuven, Belgium). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti- rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Amersham Pharmacia Biotech.

Glycosaminoglycans (GAG) Purification and Quantitation—Human wound fluids routinely collected within 24 h of surgery were centrifuged at 3000×g for 10 min to remove cells and debris. The use of this discarded material was approved by the Human Research Committee of the Brigham and Women’s Hospital (Boston), protocol number 92-5416-4. Supernatants or proteoglycans were purified from supernatants or, alternatively, the conditioned media were mixed 1:1 with buffer A and incubated with buffer A pre-equilibrated QAE-Sephadex A-25 beads overnight at 4 °C. Following incubation, beads were washed sequentially with buffer A, buffer B (300 mM NaCl, 2 mM urea, 0.5 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, 50 mM sodium acetate, pH 4.5), and bound wound fluid GAG (proteoglycans or free GAG) eluted with buffer D (2 M NaCl, 2 mM urea, 0.5 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, 50 mM sodium acetate, pH 4.5). Eluted material was precipitated with 3 volumes of 95% ethanol containing 1.3% K2SO4 and stored at −70 °C. In some WF GAG isolations, proteoglycans were further purified by boiling 10 min in 4 mM guanidine HCl buffer containing 1% Triton X-100, 50 mM sodium acetate, pH 4.5, followed by cesium chloride density gradient separa- tion. The proteoglycan-containing fraction was then precipitated with 3 volumes of 95% ethanol containing 1.3% K2SO4, reconstituted in distilled H2O, and stored at −70 °C. In some WF GAG isolations, prot eoglycans were purified from supernatants or, alternatively, the conditioned media of the A431 keratinocyte cell line by a modified version of the above anion exchange procedure. Briefly, supernatants or conditioned media were mixed 1:1 with buffer A and incubated with buffer A pre-equilibrated QAE-Sephadex A-25 beads overnight at 4 °C. Bound material was washed 3× for 5 min with buffer B and proteoglycans were eluted with buffer D. Eluted material was precipitated with 3 volumes of 95% ethanol containing 1.3% K2SO4, reconstituted in distilled H2O, and stored at −70 °C. Sulfated GAG was measured in WF GAG samples using the sulfate-binding cationic dye, dimethylmethy lene blue, according to the Blyscan (Amersham) protocol. GAG quantitation was verified by carbazole assay which does not rely on GAG sulfa tion (37). To measure the amount of dermatan sulfate present in wound fluid, WFGAG was digested 1 h with 2000 units of chondroitinase B in 50 mM Tris containing 50 mM NaCl, 4 mM CaCl2, pH 8.0. Undigested material was then precipitated with 3 volumes of 95% ethanol containing 1.3% K2SO4 and reconstituted in distilled H2O. GAG content was determined using the Blyscan Proteoglycan and GAG Assay System. For measurement of the contribution of dermatan sulfate and heparan sulfate to FGFR-2 responsiveness, WFGAG was digested for 1 h under identical conditions with chondroitinase B or 6 milliliters of heparinase in 50 mM Tris containing 4 mM CaCl2, pH 7.0.

F32 Cell Culture and Proliferation Assay—F32 cells, which express FGF receptor-1 (FGFR1), require interleukin-3 or heparin and FGF-2 for proliferation, and lack detectable heparan sulfate (38), were cultured in T75 tissue culture flasks (Falcon; Becton Dickinson Labware, Bedford, MA) with RPMI 1640 media (Cellgro; Mediatech Inc., Herndon, VA) containing 10% calf serum, 10% F10X35 cell-conditioned media (a source of interleukin-3, and supplemented with leupeptin and penicillin/streptomycin. Prior to proliferation assays cells were washed 3 times with the above media lacking interleukin-3. Cells were then added at a concentration of 2×105 cells/well to 96-well tissue culture plates (Falcon) in the absence or presence of 100 pg FGF-2 and test agents for 48 h at 37 °C in a 5% CO2, 95% air incubator. Following incubation, supernatant 6 h or overnight with WFGAG ([3H]thymidine and harvested using a TOMTEC Mach3–96 plate reader (Wallac). Membranes were then incubated overnight at 4 °C with primary antibodies, washed 3× for 5 min with Tris-buffered saline containing 0.3% Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. Primary and secondary antibodies were diluted in Blotto containing 0.3% Tween 20. Membranes were washed as above, and horseradish peroxidase was detected using enhanced chemiluminescence reagent (1.25 ml 3-aminophthalhydrazide, 0.2 ml coumaric acid, 0.3 ml hydrogen peroxide in 0.1 ml Tris, pH 8.5). Membranes were then exposed on Kodak X-Omat AR x-ray film (Eastman Kodak) and results scanned using the ScanJet 4C7 running the DeskScan II 2.3 software package (Hewlett-Packard).

Affinity Co-electrophoresis (ACE)—Binding of dermatan sulfate (Sig ma) or heparin to G2-15 (FGFR-2) was determined by ACE following derivatization of heparin with tyramine, radiolabeling, and chromatography on Sephadex G-100 to produce a low molecular weight fraction (M, ≤6000) as described previously (39). Dermatan sulfate was prepared by alkaline cleavage to remove attached peptides (1 mg of GAG in 100 μl of 10% ethanol containing 0.17 μl KOH for 1.5 h at 45 °C followed by adjustment of pH to 7.0), exchanged into water using a G-25 Sephadex column, and derivatized as described for heparin. Tyramine radiolabeled heparin was then diluted in Blotto containing 0.3% Tween 20. Membranes were washed as above, and horseradish peroxidase was detected using enhanced chemiluminescence reagent (1.25 ml 3-aminophthalhydrazide, 0.2 ml coumaric acid, 0.3 ml hydrogen peroxide in 0.1 ml Tris, pH 8.5). Membranes were then exposed on Kodak X-Omat AR x-ray film (Eastman Kodak) and results scanned using the ScanJet 4C7 running the DeskScan II 2.3 software package (Hewlett-Packard).

Human Wound Fluids Contain Abundant Dermatan Sulfate—Sulfated glycosaminoglycans (GAG) were purified from eight wound fluid samples and quantitated using dimethylmethy lene blue dye. GAG concentrations were high in all samples tested with a mean value of 31.7 μg/ml and a range from 26.1 to 37.7 μg/ml (Table I). In contrast, negligible GAG was detected in normal human serum from six individuals (data not shown), suggesting that wound fluid GAG (WFGAG) was generated during the response to injury. The concentration of WFGAG was independent of both the nature of surgery performed and volume of wound fluid generated. The ability of dimethylmethy lene blue to measure accurately the sulfated
GAG was verified by carbazole assay which yielded similar results (data not shown).

To determine if the GAG that predominates in skin, dermatan sulfate, was also present in a soluble form in wound fluid, WFGAG was treated with chondroitinase B to remove dermatan sulfate. Table I shows that as compared with the total amount of GAG measured in wound fluid, dermatan sulfate was always abundant and represented from 36 to 78% of the total GAG. Data shown represent maximal digestion under the above conditions as determined by separate dose response and time course determinations on these WFGAG preparations and on parallel samples of commercially purified GAG. Separate treatment of WFGAG with heparitinase demonstrated that the remaining sulfated GAG was predominantly heparan sulfate (data not shown).

**WFGAG Is Associated with Multiple Proteoglycans—**To evaluate which core proteins may be associated with the large quantity of GAG present in wound fluid, immunoblot analysis of WFGAG was performed with monoclonal antibodies specific for proteoglycan core proteins known to be expressed in skin (Fig. 1). Nonspecific binding of antibodies was evaluated with an excess of BSA, chondroitin sulfate ABC, or heparin. The cell-surface proteoglycans syndecan-1 and -4 were detected in GAG extracts from normal skin and conditioned media from cultured keratinocytes. These proteoglycans also appeared to be abundant in WFGAG, an observation consistent with prior reports of induction of these proteoglycans at cell surfaces in wounds (10, 19, 20). Although syndecans-2 and -3 were easily detected in normal skin extracts and keratinocyte-conditioned media, respectively, they were only faintly detected in wound fluid. The cell-surface proteoglycan, glypicanc, was strongly detected in WFGAG. In contrast, the keratinocyte-derived cell-surface proteoglycan, epican, was not detected.

To determine whether proteoglycans normally found associated with extracellular matrix were also present in a soluble form in wound fluid, immunoblots were probed with monoclonal antibodies against decorin, perlecan, or versican. Decorin and perlecan were strongly detected in both normal skin extracts and WFGAG (Fig. 1). Interestingly, versican, like syndecan-1 and -4, was easily detected in WFGAG but not extracts from normal skin.

**WFGAG Supports FGF-2-mediated Cell Proliferation—**To determine if soluble GAG produced during wound repair supports FGF-2-mediated cell proliferation, [3H]thymidine incorporation into F32 lymphoid cells, which lack cell surface heparan sulfate and express the FGF receptor, FGFR1 (38), was measured in the presence of FGF-2 and increasing amounts of WFGAG. The results show a dose-dependent increase in cell proliferation when cells were incubated with 100 ng FGF-2 and increasing amounts of WFGAG pooled from 10 patients (Fig. 2). Maximum proliferation occurred in the presence of ~3 μg/ml WFGAG, severalfold less than the amount of soluble GAG measured in vivo. Results were confirmed with WFGAG further purified by boiling in 4 M guanidine HCl followed by cesium chloride gradient separation (data not shown). FGF-2-mediated cell proliferation was not seen in the absence of WFGAG or exogenously added heparin. Similarly, WFGAG was not able to support proliferation in the absence of FGF-2. Thus, F32 proliferation in response to wound fluid GAG was FGF-dependent, and FGF-2 action could be mediated by GAG(s) present in wound fluid.

**Dermatan Sulfate Binds and Supports FGF-2-mediated Cell Proliferation—**The large amount of dermatan sulfate present in WFGAG led us to test whether dermatan sulfate binds FGF-2 and supports FGF-2-mediated cell proliferation. To evaluate quantitatively the ability of dermatan sulfate to bind directly FGF-2 under physiological conditions of pH and ionic strength, ACE analysis was performed. Fig. 3 depicts electro-

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**TABLE I**

GAG composition of human wound fluids

| Patient | Surgery                  | Wound fluid volume | Sulfated GAG | Dermatan sulfate |
|---------|--------------------------|--------------------|--------------|-----------------|
| 1       | Radical mastectomy       | 80                 | 39.0 ± 4.2   | 57 ± 10.2       |
| 2       | Areola reconstruction    | 180                | 25.2 ± 2.6   | 63 ± 8.1        |
| 3       | Radical mastectomy       | 180                | 28.6 ± 4.4   | 55 ± 4.3        |
| 4       | Radical mastectomy       | 45                 | 65.4 ± 8.9   | 36 ± 6.0        |
| 5       | Radical mastectomy       | 650                | 33.2 ± 2.5   | 38 ± 10.6       |
| 6       | Neck revision            | 35                 | 15.2 ± 3.2   | 56 ± 4.4        |
| 7       | Simple mastectomy        | 365                | 23.2 ± 1.7   | 78 ± 3.8        |
| 8       | Radical mastectomy       | 100                | 23.6 ± 2.4   | 53 ± 9.5        |

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**Fig. 1.** Multiple proteoglycans are released into human wound fluid. Soluble GAG purified from 10 pooled human wound fluid samples was applied to Immobilon-N membranes and analyzed by dot blot as described under “Experimental Procedures.” Data represent results for detection of soluble BSA, chondroitin sulfate ABC, heparin, and proteoglycans isolated from human skin, A431 cell-conditioned media (CM), or wound fluid GAG. Identical membranes probed with monoclonal antibodies specific for human syndecan-1 (DL-101), syndecan-2 (10H4), syndecan-3 (1C7), syndecan-4 (5G9), decorin (LF-136), perlecan (matrix mix), epican (mAb 17), glypicanc (mAb s1), or versican (12C5) are shown.
was from FGF-2 binding to dermatan sulfate. The direction of electrophoresis was from top to bottom. Dermatan sulfate was progressively shifted with increasing concentrations of FGF-2. B, low molecular weight heparin binding to FGF-2 as tested in A. Derived dissociation constants were 2.48 μM for dermatan sulfate and 344 nM for heparin, and described under “Experimental Procedures.”

A

B

5.0 3.0 2.0 1.0 0.5 0.3 0.2 0.1 0.01

FIG. 2. Human wound fluid-derived GAG supports FGF-2-mediated cell proliferation. F32 cells were incubated for 48 h with 100 pM FGF-2 in the presence of the indicated amounts of wound fluid GAG purified from 10 patients as described under “Experimental Procedures.” Proliferation of F32 cells was then measured by 6 h of [3H]thymidine incorporation (1 μCi/well). Mean values of triplicate determinations (± S.D.) representative of three experiments performed on WFGAG derived from 30 individual wound fluid samples are shown. [3H]Thymidine incorporation in the presence of all GAG concentrations (in the absence of FGF-2) was similar to that seen with FGF-2 alone (31.1 cpm). FGF-2 (100 pM)-mediated proliferation in the presence of heparin (500 ng/ml) was 7641 cpm.

DISCUSSION

Proteoglycans have been shown in vitro to influence multiple cell behaviors by binding physiologic ligands including several growth factors, cytokines, matrix proteins, cell-surface molecules, proteases, and protease inhibitors (2, 5–9). In the current study, we demonstrate that abundant proteoglycans are released during wound repair in vivo, and a large proportion of the soluble GAG in wounds is chondroitin sulfate B (dermatan sulfate). We find that wound fluid GAG (WFGAG) supports the ability of FGF-2 to signal cell proliferation and that this activity resides predominantly in dermatan sulfate. These observations support the hypothesis that proteoglycans released in response to injury function as essential components in wound healing and identify a unique role for dermatan sulfate in supporting cell proliferation in response to FGF-2.

Abundant Dermatan Sulfate Is Released into Wounds following Injury—Measurement of soluble GAG in wounds using dimethylmethylene blue dye demonstrated that a large amount of sulfated GAG was present in human wound fluids. Consistent with the large amounts of dermatan sulfate expressed in skin, treatment of WFGAG from individual patients with chondroitinase B showed that much of the soluble GAG was derma-
Several Proteoglycans Are Present in Wound Fluid—Purification of WFGAG by anion exchange can yield intact proteoglycans or free GAG. Measurement of GAG using dimethylmethylene blue is a relatively insensitive assay for soluble GAG determinations, measurements of total GAG may be underestimated due to the presence of nonsulfated GAG. Thus, we verified measurements with carbazole, a uronic acid binding molecule that does not rely on GAG sulfation level (37). Measurements by carbazole assay confirmed the large amounts of GAG released from wounds after injury. In addition, because measurements were similar by carbazole assay there was likely little, if any, nonsulfated soluble hyaluronic acid in these wound fluid GAG preparations. Since hyaluronic acid is not sulfated, this lack of detection may have reflected loss during purification by anion exchange, or alternatively, there may be negligible release into the wound environment.

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ence cell behaviors required for wound repair. Soluble GAG was derived both from cell surfaces and extracellular matrix. Thus, insoluble GAG serves as a source for release in response to injury and by release has paracrine potential at distant sites. The novel finding that dermatan sulfate released following injury supported FGF-2 activity further suggests that non-heparan sulfate proteoglycans also participate in the regulation of growth factor responsiveness. The specific proteoglycans that are responsible for supporting FGF-2 activity have not been identified, but demonstration of the presence of several core proteins indicates that numerous proteoglycans have the potential to function as a source of biologically active GAG in vivo. Further study of dermatan sulfate GAGs and their established ligands will contribute to our understanding of wound repair and related processes.

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