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FGF-10 and specific structural elements of dermatan sulfate size and sulfation promote maximal keratinocyte migration and cellular proliferation

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Abstract

Fibroblast growth factor-10 (FGF-10) is essential for epithelial development, while other members of this family, such as FGF-7, are not. FGF-10 is abundantly released into wounds following injury, and likely an essential growth factor required for this process. To evaluate how activation of this growth factor is controlled, multiple glycosaminoglycans were combined with FGF-10 assayed by measurement of the proliferation of cell lines expressing FGF receptor-2-IIIb, or keratinocyte migration in an in vitro wound repair assay. Dermatan sulfate (DS) exhibited greater potency than heparan sulfate or other chondroitin sulfates found in wounds. Structural variants of DS between 10 and 20 disaccharides containing iduronic acid showed maximal capacity to enable FGF-10 receptor stimulation. Furthermore, FGF-10 and DS markedly enhanced migration of keratinocytes in an in vitro wound scratch assay, while FGF-7 or other glycosaminoglycans did not. These data strongly suggest that FGF-10 activity is uniquely important in wound repair and that specific DS structural properties are necessary to promote FGF-10 function. These observations identify a novel interplay between DS and FGF-10 in mediating wound repair.

The fibroblast growth factor (FGF) family contributes to several vital steps of wound healing, including inflammation, repair, and regeneration. FGF-2, also referred to as basic FGF, promotes the recruitment of inflammatory cells to the wound site during the inflammatory phase of wound healing.¹ In addition, FGF-2 stimulates cellular proliferation and has been suggested to play a role in endothelial cell migration. FGF-7, or keratinocyte growth factor, was initially identified as a highly specific paracrine growth factor for epithelial cells.^{2,3} Further experiments revealed that FGF-7 expression is up-regulated following wounding in both mice and humans⁴ and topical administration of FGF-7 to wounds enhances wound closure.⁵ Similarly, FGF-10 (or KGF-2) expression increases considerably during wound repair. This up-regulation may be attributed to the observation that FGF-10 is released by activated $\gamma\delta$ T-cells, a process vital to optimal wound repair.^{6,7}

The FGF receptors are designated as FGFR1–FGFR4 and are composed of two to three extracellular immunoglobulin-like domains (Ig-domains), an extracellular acidic region, and a cytoplasmic tyrosine kinase domain.⁸ The signal transduction pathway of the splice variant of FGFR2, FGFR2-IIIb, is activated during wound repair upon its binding to either of two high

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affinity ligands, FGF-7 and FGF-10.^{4,9} This interaction promotes receptor dimerization and induces the activation of multiple signal transduction pathways, including the MAP kinase pathway.⁸ The extracellular signal-regulated kinases are among those intracellular molecules within the MAP kinase pathway responsible for the mitogenic response.⁸

As related molecules FGF-7 and FGF-10 bind exclusively to the same splice variant of fibroblast receptor growth factor receptor-2, FGFR2-IIIb, this receptor is up-regulated during wound repair and expressed solely by cells within the epithelium.⁴ The importance of this system to tissue morphogenesis was established by observations that targeted deletion of FGFR2-IIIb in mice results in severe developmental defects and embryonic lethality. Therefore, the mechanisms that control FGF-7 and FGF-10 function are likely important to processes that depend on tissue morphogenesis such as wound repair.

Morphogenesis and tissue repair are contingent upon the abundance and structure of linear carbohydrates, or glycosaminoglycans (GAGs).^{10–14} The major families of GAGs are heparan sulfate (HS)/heparin, chondroitin sulfate or dermatan sulfate (CS/DS), keratan sulfate and hyaluronan. GAGs are typically highly negatively charged and located on the cell surface or within the extracellular matrix (ECM). GAGs are the most abundant heteropolysaccharides in mammalian tissues and are composed of long unbranched polysaccharides containing repeating disaccharide units. Subsequent sulfation, epimerization, or deacetylation modifications characterize individual GAG chains necessary for their distinct activity. Incorporated into the chain are either of two modified sugars, *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc), and a form of uronic acid, such as glucuronate (GlcA) or iduronate (IdoA) that constitute individual GAG chain structure. One or both of these sugars will also contain sulfate groups, with the exception of hyaluronic acid. HS and CS/DS are assembled through a serine residue to protein cores, defining them as proteoglycans (PG).¹⁵ Sulfation of their sugar residues plays a particularly important role in HS and DS activity to promote functional interactions with potential proteins. DS is comprised of linear repeating disaccharide units of Gal-NAc and GlcA and is defined as a CS by the presence of GalNAc. However, the presence of IdoA distinguishes it from other CS molecules, making it more similar to heparin and HS molecules which also contain this residue (Figure 1A).¹⁶ However, HS is comprised of repeating disaccharide units of GlcNAc and GlcA, with the presence of the GlcNAc distinguishing HS from CS/DS (Figure 1B). These ubiquitous molecules have many essential functions which include a role in wound repair as they can influence cytokine/growth factor localization and interaction with cognate receptors, a process that is particularly relevant for the FGF family of growth factors.^{10,14,17}

Heparin and HS are best known for their ability to sustain FGF-2 signaling.^{10,18} These GAGs are believed to function as stabilizing cofactors for the interaction between the ligand and its receptor. HS can also bind FGF-2 on the cell surface to protect it from proteolytic degradation and generate a reservoir of growth factor.^{17,19,20} The loose sequence specificity for the interaction between FGF-2 and HS requires 2-*O*-sulfation and *N*-sulfation of the HS chain.¹¹ Unlike FGF-2, the role of GAGs in influencing function of members of the FGF family that interact with FGFR2-IIIb, and are therefore essential for epithelial growth and development, are less well studied. Given the influence of FGF-7 and FGF-10 on wound repair, understanding the dependence of GAGs for the activity of these growth factors is essential.

Previous studies have shown that either HS or DS can stimulate cellular proliferation via FGF-2¹¹ and FGF-7.^{16,21} Somewhat surprisingly, FGF-7 signaling was observed to be more robust in the presence of DS compared with HS. Because the GAG cofactors for FGF-10 have not been described, the purpose of these studies was to investigate the dependence of FGF-10 signaling on GAGs found in the wound. Our findings further illuminate an essential molecular

interaction that occurs during the wound repair process, and demonstrate that the CS GAG DS plays an important role in growth factor signaling.

MATERIALS AND METHODS

Reagents

DS from porcine intestinal mucosa (DS-03122) was purchased from Celsus Laboratories Inc. (Cincinnati, OH). CS A/C (~ 70% A and 30% C) (C-8529), heparin (H-3393), HS (H-9902) and chondroitinase ABC (C-3667) were purchased from Sigma (St. Louis, MO). Recombinant FGF-7 and FGF-10 were purchased from R&D Systems (Minneapolis, MN). BaF3 cells, a GAG-deficient mouse lymphocyte cell line stably transfected with FGFR2-IIIb (designated BaF/KGFR) lacking cell-surface GAG, were used to assess the interaction between FGFR2-IIIb and either FGF-7 or FGF-10. These cells were originally isolated according to an approved institutional protocol under IACUC guidelines and were selected and grown as described previously.²¹

GAG digestion and HPLC purification

GAG preparations were made as previously described.²¹ Briefly, 50 mg of lyophilized GAG was digested at 37 °C for 16 hours with 0.5 U of chondroitinase AC 500 µL of chondroitinase AC digestion buffer (50 mM Tris and 60 mM sodium acetate, pH 8.0). The preparation was boiled for 10 minutes to remove enzyme activity, filtered, and lyophilized. Samples were injected and analyzed using a Superdex peptide HR 10×300 cm column (Amersham Biosciences, Pittsburgh, PA) attached to an AKTA purifier HPLC system (Amersham Biosciences). The run profile consisted of running 50 mM phosphate, 150 mM NaCl, pH 7.0, buffer at a flow rate of 0.5 mL/minutes. Five hundred microliters fractions were collected using an automatic fraction collector. Fractions 17–35 were collected and lyophilized down to 500 µL. Fractions were dialyzed using a CE MWCO 500 or 1,000 membrane in water at 4 °C for 72 hours. The concentration of each size fraction was determined by carbazole assay.²¹

GAG desulfation

GAG was desulfated as previously described.^{21,22} Briefly, 100 mg of GAG was subjected to cation exchange using the Dowex 50 WX8 (Bio-Rad, Hercules, CA) and lyophilized. The sample was then divided, resuspended in of 90% Me₂SO, 10% H₂O, and boiled for 6 hours. The samples were then diluted and desalted with PD-10 columns (Amersham Biosciences). Each column was eluted with 3.5 mL of 10% EtOH, and the samples were lyophilized. The desulfated GAGs were then subjected to digestion, HPLC purification, and dialysis as described above.

Cellular proliferation assay

BaF/KGFR cells were selected for 1 week in FGF-1 and heparin as previously described.¹⁶ After selection, BaF/KGFR were expanded in T75 flasks and allowed to sit for 3 days before they were used for experiments. The growth medium used was composed of RPMI 1640, 10% fetal calf serum (FCS), L-glutamine (2.92 mg/mL), penicillin/streptomycin (100 U/mL and 50 µg/mL, respectively), and 10% interleukin-3-conditioned medium. Before experiments, cells were washed three times in 50 mL of phosphate-buffered saline (PBS) to remove any residual growth medium before seeding cells at a final concentration of 5000 cells/well. Experiments were conducted in 384-well plates (Costar, Fisher Scientific, Pittsburgh, PA) with a final volume of 25 µL/well. FGF-7 and FGF-10 were used at a final concentration of 5 ng/mL and added to the cells before seeding them in the plate. All dilutions of GAG preparations and cells were done in nonconditioned medium (RPMI 1640, 10% FCS, L-glutamine, penicillin/

streptomycin) without proliferative factors. Cells were incubated for 3 days at 37 °C before performing a nonradioactive cell proliferation assay (Promega Corp., Madison, WI).

In vitro keratinocyte wound assay

The capacity of normal human keratinocytes to migrate, proliferate, and close a defined area of injury to a confluent monolayer in vitro was evaluated by modification of a previously reported assay.²³ Primary human keratinocytes (NHEK) were grown to confluency in 24-well plates at 37 °C in serum-free Epilife® medium (Cascade Biologics, Portland, OR) containing 0.6 mM Ca²⁺, 1× Epilife® defined growth supplement, 50 U/mL penicillin, and 50 µg/mL streptomycin under standard culture conditions. On the day of the experiment, the cells were washed with 1×PBS and pretreated with mitomycin C (10 µg/mL, Sigma) 2 hours before wounding to prevent cellular proliferation. After 2 hours, the mitomycin C was removed and cells were washed three times with 1×PBS. Cells were then scratched once per well vertically with a P1000 pipette tip to create an artificial wound. Cells were then washed twice to remove cellular debris. To standardize the position of the wound for photography, small lines were made on the underside of the well using a thin permanent marker. Cells were incubated with nine different culture conditions in quadruplicate wells. Cells were stimulated with DS (2 µM) alone, HS (2 µM) alone, FGF-10 (10 ng/mL) alone, FGF-7 (10 ng/mL) alone, DS and FGF-10, DS and FGF-7, HS and FGF-10, or HS and FGF-7. PBS was used as a negative control (untreated cells). The same fields were photographed every 8 hours for a total of 24 hours and the area migrated by keratinocytes was calculated using Image J Software (NIH Image). The percent migration at 24 hours was calculated by subtracting the total area of the scratch at 24 hours from the total area of the scratch at 0 hours, divided by the total area at 0 hours multiplied by 100. Images of the areas were collected with an Olympus MVX10 microscope equipped with a DC71 camera (Olympus, Center Valley, PA). This experiment was repeated three times with the mean of all three experiments shown.

RESULTS

DS promotes FGF-10-dependent cell proliferation exceeding HS

The efficacy of DS to promote cellular responses in wounds has not been extensively studied, although previous work has demonstrated that DS, but not HS or CS, potentiated the activity of FGF-7.^{11,16} Newborn mice deficient in FGF-10 display a pronounced phenotype that includes defects in epidermal morphogenesis, indicating the importance of FGF-10 for epidermal development.²⁴ In contrast, FGF-7 null mice are viable and produce a more subtle phenotype that suggests a deficiency in hair development, but does not reveal abnormalities in epidermal growth or wound healing.²⁵ Thus, we asked if DS, HS, and heparin influence FGF-10 function and, therefore, are also fundamental to epithelial function and regulation of cellular proliferation. BaF3/KGFR cells were used as a model system to specifically study the interaction between FGFR2-IIIb and FGF-10, as compared with FGF-7.¹⁶ In the presence of FGF-10, DS promoted greater cell proliferation compared with HS and the degree of proliferation approached that observed with heparin (Figure 2A). Consistent with previous results, FGF-7 was also most active in the presence of heparin or DS compared with HS (Figure 2B).

DS oligosaccharide length influences FGF-10-dependent cell proliferation

To determine the optimal DS oligosaccharide size required for promoting FGFR2-IIIB receptor activation, high molecular weight porcine DS preparations, primarily 4-*O*-sulfated, were partially digested with chondroitinase AC to cleave the GlcA bonds, enriching for DS sequences containing IdoA. Each DS fraction (2 µM) was added to BaF/KGFR cells in the presence or absence of FGF-10 or FGF-7, respectively. The smallest DS oligosaccharide sufficient to promote at least a mean twofold increase in cell proliferation in the presence of

FGF-10 was a decasaccharide, although optimum activity was attained with somewhat longer oligomers up to a 20-mer (Figure 3A). Similar results were obtained from identically sized DS and CS oligomers in the presence of FGF-7 (Figure 3B).

IdoA is required for FGF-10-dependent cell proliferation

The epimerization of GlcA to IdoA is a defining characteristic of DS compared with other CSs and a structural characteristic shared with Heparin and HS.¹⁵ To assess the requirement of this IdoA residue for FGF-10-dependent cell proliferation, size-fractionated DS or CS A/C oligosaccharides were incubated with BaF/KGFR cells in the presence or absence of FGF-10. IdoA-containing 16-mers derived from DS preparations exhibited greater activity in promoting FGF-10-dependent cell proliferation than did the CS-derived oligomers (Figure 4A). Analysis of CS and DS 12- and 10-mers, or in the presence of FGF-7, also demonstrated that only DS GAGs could stimulate cell proliferation (Figure 4B and data not shown).

Sulfation of DS oligosaccharides is required for FGF-10-dependent cell proliferation

GAG sulfation is known to influence several biological activities.^{15,17,21} To determine if DS-dependent FGF-10 activity also requires sulfation, crude porcine DS of predominantly 4-*O*-sulfation was desulfated,²¹ digested, and purified by size exclusion chromatography as described. Cell proliferation was compared between sulfated and desulfated DS, in the presence of FGF-10 (Figure 5A). Desulfated DS fractions varying in length from two to 20 oligosaccharides exhibited no activity in promoting FGF-10-dependent cell proliferation. Analysis of a sulfated and desulfated 16-mer is shown. Similar results were obtained in the presence of FGF-7 (Figure 5B).

DS augments the capacity of FGF-10 to promote maximal keratinocyte migration

A major hallmark of keratinocytes during wound healing is their ability to proliferate and migrate to allow for reepithelialization. To assess whether DS would influence the activity of FGF-10 to enhance keratinocyte function, we employed an in vitro wound scratch assay. The ability of keratinocytes to migrate and reepithelialize a defined cell-free area was monitored every 8 hours for a total of 24 hours following wounding by a scratch. Primary keratinocytes stimulated with both DS and FGF-10, at the same dose that resulted in maximal proliferative capacity of BaF3/KGFR cells, exhibited maximal migration of over 55% over a 24-hour period compared with either DS or FGF-10 alone (Figure 6A). Furthermore, keratinocytes stimulated with either HS alone or HS and FGF-10 exhibited minimal migration comparable to untreated cells. Most notably, although we did observe a trend toward an increase in keratinocyte migration in the presence of DS and FGF-7, the effect was significantly less robust compared with DS and FGF-10 together. Photographs of un-stimulated and keratinocytes stimulated with DS alone, FGF-10 alone, DS and FGF-10, or DS and FGF-7 are shown after 24 hours following in vitro scratch injury (Figure 6B).

DISCUSSION

The importance of FGF-10 to epithelial development and wound repair has been demonstrated by the effects observed when its receptor, FGFR2-IIIb, is absent. Systemic FGFR2-IIIb null mice are viable until birth, but exhibit severe developmental malformations in cardiac,²⁶ limb,²⁷ bone,²⁸ lung,²⁷ tooth,²⁷ and thymic²⁹ development. These mice also displayed severe epidermal hypoplasia within basal keratinocytes, suggesting an important role for this receptor in keratinocyte migration, proliferation, and differentiation, particularly during wound repair.³⁰ Collectively, these defects suggest a critical role for FGFR2-IIIb in mesenchymal-epithelial signaling during early organogenesis³¹ and demonstrate the necessity for normal function of the ligands for this receptor. It was not previously known which cofactors are involved in FGF-10-mediated epithelial growth. In this paper we demonstrate that the function of ligands

through FGF-10 is dependent upon cutaneous GAGs produced during injury. The encrypted information that DS releases from the dermis as a cofactor for FGF-10 emphasizes that the extracellular regulation of FGF-10 is an essential element in wound repair.

Although FGF-7 null mice exhibited no obvious abnormalities at birth, they do develop a matted appearance by about 2 months of age.²⁵ Despite defects in hair development, FGF-7 null mice did not reveal any abnormalities in epidermal growth or wound healing. In contrast, mice deficient in FGF-10 display a phenotype that is comparable to that observed in FGFR2-IIIb mice. For instance, mice null for FGF-10 died after birth due to the abrogation of lung development, and were devoid of fore- and hind-limbs, thyroid, pituitary, and salivary glands.^{32,33} FGF-10 null mice also exhibited defects in whisker formation³⁴ and cutaneous epidermal differentiation resulting in hypoplasia.²⁴ Because FGF-10 mice die shortly after birth, it is not possible to directly assess wound healing abnormalities in these mice. Nonetheless, it is known that the resident gd T-cells within skin epidermis are a robust supply of FGF-7 and FGF-10 and are essential for normal wound repair.⁶ Studies in mice deficient in dendritic epidermal T-cells displayed delayed keratinocyte proliferation and reepithelialization, which correlated with a lack of FGF-10 expression in the epidermis.⁶ On the contrary, FGF-10 expression was detected in wild type and FGF-7 null mice. These results suggest a redundancy between epidermal FGFs during wound repair. Yet, the severe developmental defects observed in FGF-10 null mice, along with the healing defects observed in potent FGF-10 producing epidermal cells, indicates that FGF-10 may be a more critical FGF during wound repair compared to FGF-7. Moreover, the expression of the recently identified FGF family member, FGF-22, was also shown to be up-regulated following dermal injury in mice.³⁵ Interestingly, both FGF-10 and FGF-22 implement their functions through binding to FGFR2-IIIb and exhibit a 54 and a 40% amino acid identity with FGF-7, respectively,^{35,36} suggesting that other FGFs may also rely on DS to promote optimal cellular responsiveness. These observations emphasize the importance of understanding the cofactors involved in FGF signaling.

Previously, HS was presumed important due to the activity shown for heparin and many of the FGFs.^{10,37} Because a substantial amount of the wound milieu is composed of DS,¹¹ exceeding that of any other GAG, we hypothesize that DS may be an important cofactor involved in FGF-mediated epithelial signaling during wound repair. Because both DS and HS contain IdoA residues, DS resembles HS at this position but the presence of galactosamine defines this GAG as a CS. In these studies, we show that DS promotes FGF-10 and FGF-7 dependent cell proliferation that exceeds that of HS using a cell line designed to express a receptor selective for FGF-7 and FGF-10. Although many studies have suggested that HS can behave as a cofactor for FGF receptor binding, certain forms of heparin or HS were shown to reduce the interaction between FGF-2³⁷ and FGF-7³⁸ with their respective receptor by inhibiting receptor binding or mitogenic response, respectively, to negatively regulate the activity of these growth factors.

Our findings that DS was important for FGF-10 driven cell proliferation was not due to contaminating HS or heparin in the DS preparations. Previous analysis of this DS was determined to contain GalNAc, IdoA, and GlcA, but not significant levels of GlcNAc found in heparin or HS, thus validating the lack of residual heparin or HS in our DS preparations.¹⁶ We further identified the minimum DS size requirement for FGF-10 to be a decasaccharide, although optimum activity was achieved with slightly larger oligosaccharides up to a 20-mer. Each fraction was composed of primarily one size of DS that was confirmed by a predicted assessment against known standards. GAGs within the ECM are broken down into smaller fragments either by enzymatic degradation or by physical injury itself.¹⁵ Our data show that the original crude DS mixture, composed primarily of larger DS fragments, does not induce a robust proliferative response such as that observed with smaller DS oligosaccharides. Thus, these data also support a potential role for digestion of DS following injury to generate small, bioactive DS fragments derived from the wound to promote FGF-10 activity.

To assess whether the presence of the IdoA residues in DS was essential in promoting FGF activity, the capacity of CS preparations containing only GlcA residues to promote cellular proliferation was compared with IdoA containing DS preparations. Crude CS promoted little activity with either FGF-10 or FGF-7 alone; however, fractionating CS into smaller oligosaccharides only increased their activity slightly with either FGF-10 or FGF-7. Furthermore, although the CS preparation was less sulfated compared with the DS preparation and consisted of ~4% unsulfated, 56% 4-*O*-sulfated, and 40% 6-*O*-sulfated CS disaccharides, even a more highly sulfated CS preparation did not have an effect on proliferation (data not shown). Therefore, the data suggests specificity toward the IdoA residue in FGFR2-IIIb-mediated cell proliferation.

FGF-10 activity was found to be dependent on the sulfation of DS using preparations of DS that were first desulfated and then further fractionated. Fractions devoid of sulfation lost their ability to induced FGF-dependent cell proliferation. Previously, a highly monosulfated DS (90% 4-*O*-sulfated) was found to have activity in the presence of FGF-7, but increasing the sulfation did not result in an increase in proliferative capacity.²¹ Hence, the sulfation pattern of DS appears to be equally important in the ability of DS to promote cell proliferation through the binding of FGF-10 and FGF-7 to FGFR2-IIIb.

The specific mechanism by which DS regulates FGF-10 and FGF-7 activity through FGFR2-IIIb during wound repair is unknown, but may involve the localization and/or stabilization of FGF-10 and FGF-7–FGFR2-IIIb, resulting in receptor dimerization and subsequent signal transduction to promote reepithelialization of the wound. Our results using an in vitro scratch assay indicates that DS acts as a cofactor for FGF-10 to augment the ability of keratinocytes to migrate, a critical process required for optimal wound repair. Keratinocyte migration and proliferation are independent of each other and contribute separately during wound repair.³⁹ Together, our results using the FGFR2-IIIb expressing cells to assess proliferation, and the keratinocyte scratch assay to assess migration, suggest that DS participates in both processes. These data also indicate that FGF-10 plays a specific role in keratinocyte migration that is separate from FGF-7. Therefore, the role for DS in enhancing FGF-10-mediated keratinocyte migration distinguishes its possible role in wound repair from that of FGF-7. This supports the previous data in which no defects in wound repair were observed in FGF-7 knockout mice, but mice deficient in FGF-10-producing dendritic epidermal T-cells exhibited delayed keratinocyte proliferation and reepithelialization.⁶ Thus, we have identified a novel function for DS to augment the activity of FGF-10 to promote keratinocyte migration that exceeds that of FGF-7. Collectively, this emphasizes that DS is a required cofactor for maximal cellular responsiveness to FGF-10 during wound repair.

It must be noted, however, that in our studies we used soluble GAG as opposed to those GAGs formed as PGs bound to core proteins within the cell membrane or ECM. Characterization of GAG/PG interactions is complex due to the fact that numerous interactions are between the PG protein core and the binding protein, rather than the GAG itself.⁴⁰ Since such an extensive array of GAG/PG interactions is possible, with some GAG/PG interactions potentially involving direct contact between the GAG side chain and PG core, it makes it difficult to decipher the specific effects of the GAG side chain vs. the GAG/PG. It is possible that this discrepancy between cell/matrix bound GAG and soluble GAG may elicit diverse effects, particularly involving the FGFs. Yet, our combined results demonstrating direct effects of a heterogeneous population of DS on both cellular proliferation and migration seen only with FGF-10 suggests that a specific interaction occurs via the DS side chain and FGF-10 that is not dependent upon a PG protein core. Furthermore, it has been established that smaller DS fragments are generated following wounding, further supporting the idea that small, unbound DS moieties are responsible for the proliferative and migratory effects on keratinocytes. Further

experiments are required to determine the extent to which keratinocyte migration in vivo is dependent upon these molecules.

We propose that the small DS fragments generated following injury act in a paracrine manner on the epithelium to facilitate the interaction between FGF-10 and/or FGF-7 secreted by fibroblasts and $\gamma\delta$ T-cells with FGFR2-IIIb expressed on keratinocytes (Figure 7). Further studies are needed to assess the contribution of both DS oligosaccharide size and sulfation to the localization of FGF-7 and FGF-10 with FGFR2-IIIb in the wound, and to determine if this delicate balance is disturbed in abnormal wound repair. Nonetheless, results from such investigations can ultimately lead to new interventions to promote wound healing in diseased patients. Clinically, some studies suggest that GAGs play a role in the pathogenesis of skin disease.^{12,40} Specifically, it has been proposed that the balance of DS in nonhealing wounds may differ compared with that observed in normal healing wounds. Hence, the analysis of the levels and/or sulfation of DS in wound fluid from normal or diseased individuals represent a prospective clinical direction. Future directions could also include therapeutic treatment with DS to accelerate wound closure, improve wound breaking strength, or minimize scar formation.

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Glossary

CS, Chondroitin sulfate
ECM, Extracellular matrix
FGF, Fibroblast growth factor
FGFR, FGF receptor
GAG, Glycosaminoglycan
GalNAc, *N*-acetylgalactosamine
GlcA, Glucuronate
GlcNAc, *N*-acetylglucosamine
HS, Heparan sulfate
IdoA, Iduronic acid
KGF, Keratinocyte growth factor
KGFR, KGF receptor

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Figure 1.

GAG/PG structure for DS and HS. GAG chain assembly of both DS and HS to a PG protein core occurs at a serine (Ser) residue to generate a PG. Synthesis begins with the addition of xylose (Xyl), followed by the addition of two galactose (Gal) residues, and finally a glucuronic acid (GlcA) residue. The ultimate identity of the GAG is determined by the subsequent addition of *N*-acetylgalactosamine (GlcNAc) for DS (A) or by the addition of *N*-acetylglucosamine for HS (B). Both DS and HS undergo epimerization of selected GlcA residues to Iduronic acid (IdoA). Sulfation sites within the linkage region are designated above or below GalNAc, GlcNAc, or IdoA residues, which allows for encrypting important functional information into the GAG structure. DS, dermatan sulfate; CS, chondroitin sulfate; HS, heparan sulfate; GAG, glycosaminoglycan.

Figure 2.

DS exceeds heparan sulfate in its ability to promote FGF-10-dependent cell proliferation. (A) BaF/KGFR cells were grown in the presence of increasing concentrations of FGF-10 and 2 μ M of the following crude glycosaminoglycan preparations: (■) DS, (▲) Heparin, or (●) porcine intestine HS. Cells were cultured for 72 hours, and cellular proliferation determined following addition of tetrazolium dye at OD 570. (B) Experiment performed identically as in A with substitution of FGF-7 for FGF-10. For both experiments, data are represented as the mean $\pm$ SEM of triplicate determinations. * $p < 0.05$ vs. heparan sulfate by two-way ANOVA. DS, dermatan sulfate; FGF, fibroblast growth factor; CS, chondroitin sulfate; HS, heparan sulfate; ANOVA, analysis of variance.

Figure 3.

DS oligosaccharide size influences FGF-10 and FGF-7 dependent cell proliferation. DS preparations were partially digested with chondroitinase AC and fractionated by size exclusion liquid chromatography. Oligosaccharide size indicated on x-axis. (A) DS oligos at a concentration of 2 μ M were added to BaF/KGFR cells without (open bars) or with FGF-10 (filled bars) at a final concentration of 5 ng/mL. (B) DS oligos at a concentration of 2 μ M were added to BaF/KGFR cells without (open bars) or with FGF-7 (filled bars) at a final concentration of 5 ng/mL. Cells were cultured for 72 hours, and cellular proliferation determined following addition of tetrazolium dye at OD 570. The hatched bars represent control conditions with media alone, cells alone, cells plus 5 ng/mL FGF-10 or FGF-7, or cells plus 2 μ M crude DS. For both experiments, data are represented as the mean $\pm$ SEM of triplicate determinations. * $p < 0.01$ by two-way ANOVA and Bonferroni's posttest of identical cell proliferation in the absence of FGF. DS, dermatan sulfate; FGF, fibroblast growth factor; CS, chondroitin sulfate; ANOVA, analysis of variance.

Figure 4.

IdoA containing DS exceeds CS in potentiating FGF-10 dependent cell proliferation. Crude DS and CS preparations were partially digested with chondroitinase AC and fractionated by size exclusion liquid chromatography. Oligosaccharide size (crude vs. 16-mer) and the presence or absence of FGF is indicated along the *x*-axis. (A) Each DS or CS fraction at a final concentration of 2 μ M was added to BaF/KGFR cells at a final concentration of 5 ng/mL FGF-10. (B) Each DS or CS fraction at a final concentration of 2 μ M was added to BaF/KGFR cells at a final concentration of 5 ng/mL FGF-7. Cells were cultured for 72 hours, and cellular proliferation determined by OD 570 following addition of tetrazolium dye. For both experiments, data are represented as the mean $\pm$ SEM of triplicate determinations. **pp* < 0.01 vs. CS+FGF by Student's *t*-test. The hatched bars represent control conditions without addition of (A) FGF-10 or (B) FGF-7 and include media alone, cells alone, cells plus 2 μ M crude DS or CS, or cells plus 2 μ M CS or DS 16-mer. DS, dermatan sulfate; FGF, fibroblast growth factor; CS, chondroitin sulfate.

Figure 5.

Sulfation of DS is required for FGF-10-dependent cell proliferation. One hundred milligrams of DS was desulfated and separated by size exclusion liquid chromatography. The graph compares desulfated and sulfated DS 16-mer. (A) Each oligosaccharide at a final concentration of 2 μ M was added to BaF/KGFR cells without (open bars) or with FGF-10 (filled bars) at a final concentration of 5 ng/mL. (B) Each oligosaccharide at a final concentration of 2 μ M was added to BaF/KGFR cells without (open bars) or with FGF-7 (filled bars) at a final concentration of 5 ng/mL. For both experiments, data are represented as the mean $\pm$ SEM of triplicate determinations. * $p < 0.05$ vs. desulfated DS 16-mer with FGF by Student's *t*-test. The hatched bars represent control conditions with media alone, cells alone, cells plus 5 ng/mL FGF-10 or FGF-7, or cells plus 2 μ M crude DS or CS. DS, dermatan sulfate; FGF, fibroblast growth factor; CS, chondroitin sulfate.

Figure 6.

DS acts as a cofactor for FGF-10 to promote keratinocyte function. The percent migration of scratch wounds from three independent experiments is shown for each condition. (A) Primary keratinocytes were stimulated with PBS (untreated cells), DS alone (2 μ M), HS alone (2 μ M), FGF-10 alone (10 ng/mL), FGF-7 alone (10 ng/mL), DS and FGF-10, DS and FGF-7, HS and FGF-10, or HS and FGF-7. The graph represents the percent migration of the keratinocytes after 24 hours. Data are represented as the mean $\pm$ SEM of three individual experiments with samples in quadruplicate. * $p < 0.05$ by oneway ANOVA and Bonferroni's posttest. (B) Identical fields were photographed immediately after wounding and every 8 hours up to 24 hours to show the migration of keratinocytes into the cell-free areas of the artificial wound sites. 0 and 24 hour photographs were superimposed to demonstrate the difference in cell migration. The black dotted line represents the area of the scratch at $t=0$ hours. The white dotted line represents the area of the scratch at $t=24$ hours. The mean percent migration after 24 hours is indicated in the lower left hand corner. DS, dermatan sulfate; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; HS, heparan sulfate; ANOVA, analysis of variance.

Figure 7.

Proposed model for the function of DS in FGF-10 signaling through FGFR2-IIIb in wound reepithelialization. (A) In noninjured skin, $\gamma\delta$ T-cells and fibroblasts reside in the epidermis and dermis, respectively. (B) In keratinocytes, the splice variant of FGFR2, FGFR2-IIIb, exists as a monomer consisting of three Ig loops, and acid-box region, a transmembrane domain (TM), and two intracellular tyrosine kinase domains (TK1 and TK2). (C) Following injury, resident $\gamma\delta$ T-cells and fibroblasts secrete FGF-10 into the wound, while small fragments of DS are released into the wound from the extra-cellular matrix. (D) The increase in FGF-10 is then enabled by DS to act in a paracrine manner on keratinocytes through FGFR2-IIIb. The presence of small DS oligosaccharides facilitates the interaction between the FGFs and FGFR2-IIIb to promote receptor dimerization. Ultimately, the receptor is phosphorylated and intracellular signal transduction pathways are activated, stimulating wound reepithelialization by keratinocytes. DS, dermatan sulfate; FGF, fibroblast growth factor.