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Heparin-Degrading Sulfatases in Hepatocellular Carcinoma: Potential roles in Pathogenesis and Identification of Therapeutic Targets

Jin-Ping Lai, M.D., Ph.D., James R. Thompson, Ph.D., Dalbir S. Sandhu, M.B.B.S., and
Lewis R. Roberts, M.B.Ch.B., Ph.D.

Center for Basic Research in Digestive Diseases, Miles and Shirley Fitterman Center for Digestive
Diseases, Mayo Clinic College of Medicine and Mayo Clinic Cancer Center, 200 First Street, SW,
Rochester, Minnesota, 55905. Telephone 507-538-4877. Fax 507-284-0762.

Abstract

Hepatocellular carcinoma (HCC) is often diagnosed at an advanced stage for which there are limited treatment options. Two recently identified human heparin-degrading endosulfatases, named sulfatase 1 (SULF1) and sulfatase 2 (SULF2), have been found to be involved in liver carcinogenesis. SULF1 and SULF2 desulfate cell surface and extracellular matrix heparan sulfate proteoglycans (HSPGs) and modulate heparin-binding growth factor signaling in multiple cancers, including HCCs. SULF1 inhibits HCC tumor cell growth *in vitro* and in nude mice *in vivo*, partially through effects on gene expression mediated through histone H4 acetylation. While SULF1 is downregulated in the majority of HCC cell lines and approximately 30% of primary HCCs, SULF2 is up regulated in almost all HCC cell lines and in 60% of primary HCCs. In contrast to the tumor suppressor effect of SULF1, expression of SULF2 activates mitogen activated protein kinase (MAPK) and Akt pathways, promotes HCC cell growth *in vitro* and *in vivo*, and is associated with decreased survival of HCC patients. Targeting SULF2 or the interaction between SULF2 and SULF1 may lead to novel therapeutics for the treatment of HCCs.

Keywords

Hepatocellular carcinoma (HCC); SULF1; SULF2; heparan sulfate proteoglycan (HSPG); heparan sulfate glycosaminoglycans (HSGAGs); Glypican 3 (GPC3); heparin-binding growth factor; MAPK kinase; Akt

INTRODUCTION

It is estimated that 626,000 patients are diagnosed with hepatocellular carcinoma (HCC) every year and it is the third most common cause of cancer related death, accounting for 598,000 deaths annually worldwide[1]. Although HCC is not common in developed countries, its incidence has been increasing in the United States, Japan, and western Europe[2,3]. In the United States, HCC ranks as the 10th most common cause of death from cancer, with the age-adjusted incidence more than doubling between 1985 and 2002[2,4,5]. This increase is projected to continue for 20 to 30 years as a long term consequence of increased rates of hepatitis C virus (HCV) infection between 1960 and 1990[2,3,6].

Address for correspondence: Lewis R. Roberts, MD Associate Professor of Medicine Mayo Clinic College of Medicine Miles and Shirley Fitterman Center for Digestive Diseases 200 First Street SW Rochester, Minnesota 55905 Tel: 507 538-4877 Fax: 507 284 0762 roberts.lewis@mayo.edu .

Important risk factors for HCC are chronic hepatitis and cirrhosis due to hepatitis B and C virus infections, alcohol, non-alcoholic steatohepatitis, other causes of chronic liver disease, and dietary exposure to fungal aflatoxins[6]. Although effective surgical and local therapies are available for early HCCs, advanced HCCs are resistant to conventional chemotherapy[7]. Improvements in our understanding of the molecular pathogenesis of HCC have allowed the development of sorafenib, a multikinase inhibitor which is the first agent shown to be effective in advanced HCC. Further advances should lead to additional new therapeutic strategies against HCC.

SULF 1 and SULF2 belong to a new family of sulfatases which desulfate heparan sulfate proteoglycans

The activities of two recently identified extracellular sulfatases have profound and differing effects on HCC. Designated sulfatase 1 (SULF1) and sulfatase 2 (SULF2), these heparan sulfate 6-O-endosulfatases hydrolyze sulfate ester bonds of heparan sulfate glycosaminoglycans (HSGAGs). HSGAGs are linear polysaccharides of about 50-200 disaccharide repeats with regions of glucuronic acid-*N*-acetylglucosamine and regions of 2-O-sulfated iduronic acid-*N*-sulfoglucosamine (S-domain) interspaced by transition zones containing both sulfoglucosamine and acetylglucosamine, which are covalently attached to a polypeptide core to form heparan sulfate proteoglycans (HSPGs). The disaccharide units can be sulfated at the 2-O position of the uronic acid and the N-, 3-O and 6-O positions of glucosamine[8,9]. Dynamic changes in this non-random pattern of sulfation correlate with and are important to a number of extracellular signaling pathways essential for cell function and development. The initial presumption was that SULF1 and SULF2 would have similar and complementary functions with regard to HCC, for example, both quail SULFs display the same *in vitro* specificity toward trisulfated disaccharides[10]. However, mouse and human SULF1 and SULF2 appear to have unique structural features that give rise to altered ligand specificities *in vivo*, specificities not restricted simply to S-domains as for quail SULFs[11]. These structural differences translate into important functional differences in the effects of SULF1 and SULF2 in HCC. SULF1 promotes HCC cell apoptosis by decreasing heparin-binding growth factor signaling and also potentiates the therapeutic effects of histone deacetylase inhibitors against HCC *in vitro* and *in vivo* through a mechanism involving increased histone H4 acetylation[12,13]. In contrast to the tumor suppressor effect of SULF1, SULF2 promotes tumorigenesis of HCC in nude mice. SULF2 expression also corresponds with more rapid recurrence and shorter survival of patients with HCC after surgical resection.

In this review, we present recent studies on the roles of SULF1 and SULF2 in HCC with particular reference to their *in vitro* and *in vivo* effects on cellular growth and survival, and on tumor proliferation, migration, invasion, and angiogenesis. We then present a proposal that SULF2 appears to be an excellent target for future therapeutic development.

SULF1 and SULF2 domain structure, substrate specificity and redundancy

There are 17 human sulfatase genes, which are dispersed throughout the genome [10,14,15]. SULF1 and SULF2 are unique in their action as endo-enzymes, eliminating intra-polymer sulfate groups from the 6-O position of glucosamine within intact heparin, and in their ability to modulate interactions of heparan sulfate proteoglycans with extracellular signaling proteins[14]. Human SULF1 and SULF2 have 64% sequence identity[14,16] and are roughly 870 amino acids in length. In contrast, other sulfatases are 380-500 residues in length. Both SULFs are modified by asparagine-linked glycans before secretion, perhaps at 6-12 sites, and various varieties have been discovered in cell medium[14]. *N*-linked glycosylation of quail SULF1 appears essential for secretion, membrane targeting, and

enzyme activity[17]. Little conservation exists among potential glycosylation sites between species.

Two primary regions exist in SULF1 and SULF2 (Figure 1). Amino acids 25 to roughly 400 of mature SULF (human SULF1 numbering) comprise the enzymatic “sulfatase domain” conserved among sulfatases. This catalytic region of SULF1 and SULF2 display 80% identity. A post-translationally modified cysteine forms a C-formylglycine within the active sites of both enzymes[18] that is involved in hydrolytic cleavage of sulfates from the 6-O position of glucosamines in substrates[19-24]. Theoretical models of the catalytic cores of SULF1 and SULF2 can be built using 13 homologous crystallographic structures at present. A shallow groove at the active site is seen without features to support interactions with HSGAGs, and one hypothesis is the pocket binds little beyond a 6S glucosamine. Between SULF1 and SULF2 eleven residues within 5 Å of the C-formylglycine are equivalent, and it must be inferred that the molecular basis for ligand specificity lies elsewhere.

Amino acids ~402 to 871 encompass a carboxy-terminal extension that demarks SULF1 and SULF2 from other sulfatases. Predictions are this polypeptide is for the most part structured though no experimental structure exists. Twelve conserved cysteines exist implying disulfide bonds may be present. The sequence among species diverges most greatly between residues 457 to 613. Overlapping residues 400 to 490 are probably α -helices and clearly non-homologous with the four-stranded β -sheet and α -helix that follow the catalytic core in other arylsulfatase structures. Instead, a region in both SULFs between residues 765 to 835 is expected to form a subdomain of four α -strands followed by an α -helix. The Phyre modeling server[25] aligns this segment with the similar conserved tertiary structure in other family structures (i.e. residues 464-516 in PDB ID 1hdh). Predictions point to two segments of coiled-coil structure between residues ~643 to 670 and ~712 to ~732 which are of unknown significance. Numerous lysines and arginines suited for interactions with sulfated HSGAGs are found within 402-871, particularly the human SULF1 R₇₂₁RRKKERKEKRRQRK₇₃₅ and SULF2 K₄₀₂KKMR₄₀₆, R₅₁₈RKKLFFKKKYK₅₂₈, K₆₅₃KKR₆₅₆ and K₇₀₂RKKKLRKLLKR₇₁₃ sequences that fit the heparin-binding motif BBXB (B is K or R and X any amino acid). Quail SULF1 fails to bind cell surfaces when amino acids 418-736 are deleted[26]. Quail SULF1 is not released from cell surfaces using heparin or heparitinase[26] and it binds mutant CHO cells defective in glycosaminoglycan synthesis[27]. When six similar heparin-binding motifs were deleted from quail SULF2, the enzyme largely failed to bind cell surfaces and HSGAGs and lost 96% of wild-type activity[10]. 85% of which quail SULF2 activity was lost by deleting RRRKKKLRKLLKRIK, aligns with human SULF2 K₇₀₂RKKKLRKLLKR₇₁₃. Finally, residues 736 to 843 have significant homology to glucosamine 6-sulfatase and N-acetylglucosaminyltransferase and may convey specificity toward glucosamine and GlcNAc in both exo- and endosulfatases[14].

Quail SULF1 and SULF2 have been suggested to be redundant enzymes with similar specificity *in vitro* towards trisulfated IdoA2S-GlcNS(6S) within the S-domains of HSGAG[10]. Mammalian SULF1 and SULF2 may behave differently *in vivo*. The disaccharide content has been compared between radiolabeled HSGAG purified from mouse embryonic fibroblasts and from wild-type, SULF1, SULF2, and SULF1&2 double knock-out mice[11]. The SULF1 knock-out showed an increase in UA-GlcNAc(6S) specific to transition zones, UA(2S)-GlcNS(6S) specific to S-domains and UA-GlcNS(6S) present in both transition zones and S-domains when normalized to wild-type. The SULF2 knock-out demonstrated an increase in UA-GlcNAc(6S) but a distinctly different decrease in the UA(2S)-GlcNS(6S) and UA-GlcNS(6S) disaccharides isolated[11]. It was then found that SULF1 expression is up regulated in the cells lacking SULF2, but SULF1 loss is not compensated by more SULF2 expression[11]. These results may suggest that differences in

ligand specificity exist but more experimentation is required. The differences found within the carboxy-terminal domains may explain the different functions of the SULFs in human carcinogenesis.

SULF1 downregulates HSPGs and suppresses heparin-binding growth factor signaling in HCC

Cloning of SULF1

The human SULF1 sequence was initially identified in a screen for long coding sequence genes and designated KIAA1077. Through suppression subtraction hybridization analyses of two early and late stage tumors subtracted against normal ovarian epithelial brushings, the mRNA corresponding to SULF1 was shown to be down-regulated in 75% of ovarian cancer specimens[28]. Subsequent examination of SULF1 expression in other cancer types has shown complete loss or markedly diminished expression of SULF1 in cancer cell lines derived from breast, pancreas, kidney and primary liver cancers, suggesting that down regulation of SULF1 is relatively widespread among epithelial cancers[29]. Separately, Morimoto *et al.* assembled a 257-kb genomic contig from clones that contained the entire SULF1 gene. Using the GeneScan exon prediction program in conjunction with EST data, they identified a 232-bp sequence within the putative first exon encoding a signal sequence related to the quail SULF1 protein. A PCR-based pool selection technique was applied to a human prostate “Cap-Finder” cDNA library, leading to the identification of a 4669-bp cDNA. The cDNA was fully sequenced on both strands, confirming the sequence derived by genomic analysis[14]. Interestingly, the sequences identified by Shridhar *et al.* from tumors and Morimoto *et al.* differed by the presence of two noncoding exons, one 5' to the exon with the signal sequence (314 bases, designated exon 1)[29] and another 3' to this exon (165 bases, designated exon 2)[29].

SULF1 expression in HCC

It appears that the frequency of loss of SULF1 expression in cancer cell lines is higher than in the corresponding primary tumors. For example, SULF1 mRNA was undetectable in 9 of 11 (82%) hepatocellular carcinoma cell lines examined (Figure 2A). However, the mRNA was down regulated in only 29% of resected primary tumors. This discrepancy could possibly be due to selection bias as resected cancers in general tend to be lower stage, lower grade cancers than those cancers that are capable of being established as *in vitro* cell lines; on the other hand, it is possible that loss of SULF1 is important for autonomous *in vitro* growth of HCC cell lines, while high levels of SULF1, even though they are oncogenic *in vivo*, do not allow development of cell lines. Lower SULF1 expression may therefore correlate with a more aggressive cancer phenotype[12].

Loss of SULF1 occurs partially through DNA methylation

SULF1 appears to be dysregulated by both genetic and epigenetic mechanisms in HCCs. 22 of 52 (42%) of primary HCCs showed loss of heterozygosity (LOH) of polymorphic markers at the SULF1 locus. Examination of SULF1 gene copy number using fluorescence in situ hybridization (FISH) showed intact gene copies at the SULF1 locus in 4 HCC cell lines examined, two of which do not express SULF1. Two or three copies of the SULF1 locus were found in the SULF1 negative HCC cell lines SNU449 and SNU387, while four or five copies were found in SULF1 positive HCC cell lines SNU182 and SNU475 (Figure 2B). The retention of the SULF1 locus in the non-expressing cell lines suggests that the gene is repressed by epigenetic mechanisms. Subsequently, treatment with the demethylating agent 5 aza-deoxycytidine led to re-expression of SULF1 in 3 of 5 SULF1-negative HCC cell lines tested, including the SNU449, SK Hep1, and Huh7 cell lines, but not the HepG2

and Hep3B cell lines (Lai et al, unpublished data). This strongly suggests that either SULF1 or a SULF1-regulating gene is down-regulated by epigenetic DNA hypermethylation[12].

SULF1 desulfates HSPGs and down regulates heparin-binding growth factor signaling pathways

Effects of SULF1 on HSPG sulfation

Initial studies of the quail and drosophila homologs of SULF1 suggested that they were localized at the cell surface. Dhoot *et al.* proposed that quail sulfatase 1 (qSulf1) might be involved in desulfation of cell surface HSPGs, the major sulfated macromolecule at the cell surface, and consequently in regulation of HSPG-mediated growth factor signaling[26]. A number of direct and indirect sources of evidence have provided substantial support for this hypothesis. To determine whether SULF1 expression causes desulfation of cell surface HSPGs, Lai *et al.* performed immunocytochemistry using the 10E4 anti-HSPG monoclonal antibody that recognizes native heparan sulfate containing mixed *N*-sulfated and *N*-acetylated glucosamine moieties[32-35]. The HCC cell line SNU182, which expresses a high level of endogenous SULF1, was compared with SNU449 or vector-transfected SNU449 cells, which do not express SULF1, and to three stably-transfected SNU449 clones expressing full-length SULF1. The low SULF1-expressing parental SNU449 and SNU449-Vector cells showed cell surface staining for *N*-sulfated glucosamine-containing HSGAGs; in contrast, cell surface staining by 10E4 was substantially diminished or absent in the high-SULF1 expressing SNU182 cell line and in all three SNU449-SULF1 clones[12]. As a control to confirm the presence of HSPGs on the surface of all the cell lines, Lai *et al.* used the 3G10 “anti-stub” HSGAG monoclonal antibody after heparitinase treatment; this antibody recognizes the HSGAG neo-epitope produced by digestion of HSGAGs with heparitinase, and showed the presence of HSPG stubs on both sulfatase-expressing and sulfatase-negative cell lines, confirming that SULF1 does not act by inhibiting HSGAG production or transport to the cell surface. Transient expression of a construct expressing antisense SULF1 mRNA restored the cell surface 10E4 anti-HSPG immunoreactivity of the SULF1-positive cell lines. These and similar results from other groups strongly suggest that SULF1 desulfates HSGAGs at the cell surface[12,36]

Effects of SULF1 on FGF receptor tyrosine kinase signaling

It is now clear that HSPGs serve as key regulators of heparin-binding growth factor signaling. The best-studied example of this regulation is the role of HSPGs in FGF-2 signaling. HSPGs serve as a co-receptor for binding of FGF-2 to its cognate FGF receptors, leading to the formation of a ternary complex that is essential for cell proliferation and angiogenesis[37,38]. Sulfation of specific sites on HSPGs is critical for this interaction. The interaction of FGF-2 with FGFR and HSPGs leads to receptor dimerization, activation and autophosphorylation. The FGFR tyrosine kinase phosphorylates tyrosines in the cytoplasmic domain of the receptor as well as in the adaptor SNT/FRS, a docking protein for initiators of intracellular signaling pathways. Consequently, FGF-2 stimulation activates downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway.

Sulfation of HSGAGs at the cell surface has been shown to be required for formation of the FGF2-HSGAG-FGFR ternary complex, which initiates FGF2 signaling[39,40]. To explore the role of desulfation of cell surface HSGAGs by SULF1 in cellular growth control, several groups have investigated the effect of SULF1 expression on FGF2 signaling in cancer cell lines, including HCC cells[12,29,36,41]. FGF2 signaling is abrogated by SULF1 expression, with subsequent inhibition of receptor dimerization and inactivation of the intracellular FGFR tyrosine kinase. Decreased phosphorylation of p44/42 (ERK1/2) MAP kinases

consequently leads to decreased cell proliferation and survival and sensitizes cells to drug-induced apoptosis[12,29,36,41].

SULF1 down regulates signaling of other heparin-binding growth factors

Like FGF2, other heparin-binding growth factors, such as hepatocyte growth factor (HGF), heparin-binding EGF (HB-EGF), and vascular endothelial growth factor (VEGF) are also postulated to play an integral role in the uncontrolled growth of HCCs. HGF (also known as scatter factor) is a stromal-derived heparin-binding pleiotropic polypeptide, and its receptor c-Met is over expressed in a variety of human cancers[42]. Binding of HGF to c-Met activates the catalytic tyrosine kinase domain of c-Met and subsequently activates the MAPK and PI3K signal pathways, contributing to the expression of the pro-angiogenic factors IL-8 and VEGF[42]. While the overexpression of HGF and c-Met is associated with tumor metastasis and angiogenesis[42,43], the mechanisms underlying these effects remain unclear. HGF has the capacity to mediate 'invasive growth', a hallmark of metastasis, due to coordinated induction of cell motility and degradation of the extracellular matrix[44,45]. While HGF-mediated activation of ERK is postulated to be important for tumorigenesis, there is a paucity of information on the activation of ERK mediated through HGF signaling in human cancers. In both HCCs and head and neck cancer cells, low SULF1-expressing cells showed increased sulfation of cell-surface HSPGs, enhanced FGF and HGF-mediated signaling, and increased HCC cell growth. Conversely, forced expression of SULF1 resulted in decreased sulfation of cell-surface HSPGs and the abrogation of HGF-mediated activation of both the ERK and PI3K/Akt pathways, with a resultant decrease in proliferation and mitogenicity *in vitro*. Concomitantly, HGF-mediated cell motility and invasiveness were attenuated in SULF1-expressing cancer cells. With the abrogation of the growth and survival signals, SULF1 expression increased the sensitivity of HCC cells to staurosporine- and cisplatin-induced apoptotic cell death. Therefore, loss of SULF1 in HCC leads to enhanced HGF signaling and activation of cell survival pathways[12,41].

Similar effects to those of SULF1 on FGF signaling are seen with HB-EGF and VEGF signaling as well[29,46]. Given the profound effect of SULF1 in inhibiting growth and survival signaling by multiple heparin-binding receptor tyrosine kinases, HCC cells with high-level SULF1 expression were shown to be extremely sensitive to staurosporine- or cisplatin-induced apoptosis, whereas low SULF1-expressing cells were resistant. Transfection of SULF1 into SULF1-negative cells restored staurosporine and cisplatin sensitivity[12,41].

SULF1 potentiates the efficacy of HDAC inhibitors *in vitro* and *in vivo* through modulation of histone H4 acetylation

SULF1 expression significantly inhibits the growth of HCC xenografts in nude mice. Because HSPGs are expressed not only on epithelial cells, but also in the extracellular matrix, on endothelial cells, stellate cells, and other stromal cells, it is possible that SULF1 expression on the HCC cell surface has a paracrine effect and results in decreased sensitivity of endothelial and stromal cells to pro-angiogenic heparin-binding growth factors such as FGF2 and VEGF. While investigating the epigenetic regulation of SULF1, Lai *et al.* discovered that histone H4 acetylation is up-regulated by SULF1 in HCC cells. This appears to occur both through the activation of histone acetyltransferases (HATs) and through the downregulation of histone deacetylases (HDACs). Expression of SULF1 therefore results in an increase in the acetylation of histone H4 and to subsequent down-regulation of the PI3K/AKT and MAPK kinase pathways. Consequently, SULF1 potentiates the inhibition of HCC tumorigenesis by HDAC inhibitors both *in vitro* and *in vivo*. The potential exists for future translation of these effects into therapeutic strategies for HCC[13].

SULF2 has an oncogenic function opposite the tumor suppressor effect of SULF1 in HCC

Cloning of SULF2

Using the SULF1 cDNA sequence in a BLAST search, Morimoto *et al.* identified a highly related partial cDNA sequence (KIAA1247) from humans in the GenBank™ data base and assembled a 311-kb contig from genomic sequences. A full-length cDNA was predicted by exon scanning. They then used a forward and reverse primer to clone a full-length (4279 bp) human SULF2 cDNA from a human lung cDNA library. The sequence exactly matched the sequence predicted from the exons identified in the genomic contig[14].

SULF2 expression in HCC

SULF2 expression has also been determined in 11 HCC cell lines (Figure 2A). In contrast to SULF1, which is downregulated in 9 of the 11 HCC cell lines[12], SULF2 is up-regulated in 8 of the same 11 HCC cell lines[30], as compared to normal hepatocytes. Gene expression microarray analysis of HCC tumor and adjacent benign liver tissues from 139 HCC patients also showed upregulation of SULF2 in 79 of 139 (57%) of primary HCCs (Figure 3) [30,31]. Over-expression of SULF2 protein in HCC was also confirmed by immunohistochemistry using an antibody against human SULF2 (Figure 4).

Oncogenic function of SULF2 in HCC

Role of SULF2 in carcinogenesis

The functional role of SULF2 in cancer has been controversial. It has been reported that like SULF1, SULF2 also inhibits the growth of multiple myeloma cells[47]. In contrast, Morimoto-Tomita *et al.* reported that SULF2 is up-regulated in breast cancer[48] and mobilizes heparin-bound growth factors and chemokines, such as VEGF, FGF-1, and SDF-1[48]. SULF2 has also recently been shown to be mutated in breast cancer, although the functional effects of these mutations are not yet determined[49]. Silencing of SULF2 has been shown to decrease cell proliferation and survival of pancreatic cancer cells[50] and increased expression of SULF2 increased drug resistance in the MCF-7 cell line[51]. SULF2 therefore appears to act as an oncoprotein in the majority of cancer types.

SULF2 functions as an oncoprotein in HCC and predicts prognosis of HCC patients

To investigate the role of SULF2 in predicting the outcome of patients after surgical resection of HCC, Lai, *et al.* analyzed the effect of SULF2 mRNA expression on survival and recurrence after surgery in 139 patients from four different geographic regions around the world who underwent surgical resection for primary HCC. There was increased SULF2 expression in the tumor compared to the adjacent benign tissues in 79 (57%) of the HCCs. Survival analysis showed that patients with tumors expressing high levels of SULF2 had a significantly worse prognosis (Figure 5) and a more rapid rate of tumor recurrence after surgery[30]. To define the molecular characteristics of the HCCs and assess the prognostic value of mRNA gene expression profiles, Lee *et al.* analyzed the global gene expression patterns of 91 human HCCs and identified two distinct subclasses (A and B) of HCC that are highly associated with patient survival. Patients with a cluster A gene expression profile have a worse prognosis than patients with a cluster B gene expression profile[31,52]. Patients with the highest SULF2 levels were associated with the poor prognosis cluster A gene expression profile. Thus, SULF2 is frequently up regulated in human HCCs and predicts a more aggressive tumor phenotype. Examination of the list of genes that correlate most closely in expression to the tumor expression of SULF2 revealed the presence of a number of tyrosine kinase signaling and Wnt pathway genes[30].

SULF2 promotes HCC cell growth in vitro and in vivo

Of the 11 HCC cell lines examined by Lai *et al.*, Hep3B is the only cell line with almost complete absence of SULF2 mRNA expression (Figure 2A). Forced expression of SULF2 in Hep3B cells significantly increased proliferation and migration of Hep3B cells in vitro. To confirm the *in vitro* effects of forced expression of SULF2 *in vivo*, subcutaneous tumor xenografts were established in nude mice from Hep3B Vector and Hep3B SULF2-expressing cells. SULF2 expression significantly increased tumor growth, decreasing the median time to reach a tumor size of 1000 mm³ by 28 days[30].

SULF2 up-regulates glypican 3 and enhances FGF signaling in HCC

Glypican 3 (GPC3), an HSPG anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) link, has been shown to function as an oncoprotein in HCC[53-55]. Although SULF1 and SULF2 appear to have the same 6-O-endosulfatase activity on HSPGs, there must be structural differences, perhaps in the specific sulfated domains of HSPGs on which the two different sulfatases act, that result in their profoundly different effects on cancer cells. Exploration of the interaction between SULF2 and the oncogenic HSPG, glypican 3, revealed that forced expression of SULF2 in Hep3B cells enhances GPC3 expression, FGF2 binding, and FGF2 signaling, both *in vitro* and *in vivo*. Further, knockdown of SULF2 in the SULF2 over-expressing Huh7 HCC cell lines down-regulated cell membrane GPC3 expression[30]. Thus, the effects of SULF2 appear to be mediated in part through increased GPC3 expression. Based on the available evidence, we speculate that there are two different classes of sulfated HSGAGs at the cell surface or in the extracellular matrix. The first class, which functions primarily as a sulfation-dependent co-receptor for signaling by heparin-binding growth factors, is a substrate for SULF1. Enhanced activity of SULF1 consequently results in desulfation of co-receptor HSGAG moieties and abrogation of heparin-binding growth factor signaling while down-regulation of SULF1 leads to enhancement of heparin-binding growth factor signaling. In contrast, the second class of HSGAGs serves as a cell surface and extracellular storage compartment for sequestration of heparin binding growth factors. This second storage class of HSGAGs is a substrate for SULF2. Enhanced activity of SULF2 therefore results in release of heparin-binding growth factors from the HSGAG storage sites and increases the availability of growth factors for signaling. While the data for these effects is sufficiently robust for the effects of SULF1 and SULF2 on signaling by heparin-binding receptor tyrosine kinase growth factors, it is as yet unclear whether there is a difference in the actions of SULF1 and SULF2 on molecules in other signaling pathways such as the Wnt and hedgehog pathways and the possible mechanistic bases for these differences. It is possible that, in contrast to the opposing effects of SULF1 and SULF2 on heparin-binding receptor tyrosine kinase signaling, the effects on the Wnt, hedgehog or other pathways are overlapping and functionally cooperative, as has been demonstrated by analysis of embryonic, muscle and skeletal development of single and double SULF1 and SULF2 knockout mice[11,56-60].

Conclusion

Mechanistic studies of the functions of the recently identified heparin-degrading 6-O endosulfatases SULF1 and SULF2 have led to the appreciation of new paradigms of regulation of heparin-binding growth factor signaling. These new findings have significance for our understanding of the coordinated regulation of cellular sensitivity to growth factors and substantial potential for translation into clinical applications. SULF1 is down regulated in a majority of HCC cell lines and a proportion of primary HCCs. Expression of SULF1 desulfates cell surface HSPGs and sensitizes HCC cells to chemotherapy-induced apoptosis, in part through decreasing heparin-binding growth factor signaling and histone H4 acetylation. In contrast to the tumor suppressor effect of SULF1, SULF2 is overexpressed in

the majority of HCC cell lines and primary HCCs, and up-regulation of SULF2 in HCCs correlates with worse prognosis of HCC patients. SULF2 functions as an oncogenic protein in HCCs through activation of FGF signaling and the downstream AKT/MAPK pathway and also through increased expression of glypican 3 with consequent promotion of HCC cell proliferation and migration *in vitro*, and enhancement of HCC tumor growth *in vivo*. These unique observations raise important questions about the molecular mechanisms underlying the disparate effects of SULF1 and SULF2 on cell growth. Since both molecules appear to have a 6-O-endosulfatase activity on HSPGs, we hypothesize that they subserve their differing effects through the non-enzymatically active hydrophilic and C-terminal domains of the proteins, which have different structures and may target the sulfatases to different subtypes and/or subdomains of HSPGs. Experiments specifically designed to examine this hypothesis are currently in development. We propose that SULF1 desulfates HSPGs leading to decreased growth factor signaling, while SULF2 increases GPC3, releases growth factor ligands from heparan sulfate storage sites and increases growth factor binding to growth factor receptors at the cell surface (Figure 6). Future studies targeting SULF2 or the interaction between SULF2 and SULF1 may lead to the development of novel modalities for the treatment of HCCs.

Future perspective: A possible role of SULF2 or interaction of SULF2 with SULF1 in future treatment of HCC

Recent advances in cancer biology suggest that a limited number of pathways are responsible for the initiation and maintenance of dysregulated cell proliferation, which is the major cellular alteration responsible for the cancer phenotype. New agents in development target several of these critical pathways, including the MAPK pathway, the PI3K/Akt pathway, the Wnt/beta-catenin signaling pathway, and the sonic hedgehog pathway. Many of these pathways have ligands for which cell surface proteoglycans act as co-receptors. Studies of the newly-discovered sub-family of heparin-degrading endosulfatases, SULF1 and SULF2, suggest that these sulfatases can modulate heparin-binding growth factor signaling in one of two ways. First, SULF1 appears to act primarily by desulfation of HSPG co-receptors and consequent interference with formation of the ligand-receptor-HSPG complex required for efficient downstream signaling; second, SULF2 appears to desulfate HSPGs in the extracellular matrix or on the cell surface which act as a storage site for growth factor ligands and cytokines. Desulfation of these storage site HSPGs releases the growth factors, thus increasing the local concentration of heparin-binding growth factors and enhancing cell survival, growth, migration, and tumor angiogenesis. Therefore SULF1 and SULF2 appear to be uniquely situated to have profound effects on cellular responses to multiple heparin-binding growth factor signals. The relative activity of the two enzymes regulates the sensitivity of cells to the external growth factor milieu, with SULF1 decreasing growth factor responsiveness and SULF2 enhancing growth factor responsiveness. This would allow an integrated and coordinated cellular response to changes in the external environment. This unique conjunction of cell signaling mechanisms also potentially presents a very attractive set of targets for modulation of tumor growth and sensitivity to chemotherapy.

There are a number of SULF1 and SULF2 related phenomena that are as yet incompletely understood. Although SULF1 is downregulated in 82% of HCC cell lines and functional studies reliably show that it functions as a tumor suppressor, it is only a minority, approximately 30% of primary HCCs, that have downregulation of SULF1 expression[12]. The majority of HCCs have increased SULF1 mRNA expression and about a third of HCCs have very high SULF1 levels (over 4× higher than surrounding benign tissue). This suggests that although the effect of SULF1 is to inhibit the receptor tyrosine kinase pathways that are dominant in HCC cell lines, there may be other oncogenic pathways that are activated by

SULF1 in primary HCCs. In addition, SULF1 has been shown to enhance histone acetyltransferase activity and decrease histone deacetylase activity, thus enhancing the sensitivity of HCC cells to chemotherapy drugs *in vitro* and *in vivo*. The mechanistic basis for these effects has not been clarified. Finally, the mechanisms regulating expression of SULF1 and SULF2 have not been elucidated.

Executive Summary

SULF 1 and SULF2 belong to a new family of sulfatases which desulfate heparan sulfate proteoglycans

- Two recently identified human heparin-degrading endosulfatases, named sulfatase 1 (SULF1) and sulfatase 2 (SULF2), have been found to be involved in carcinogenesis.
- Sulfatases 1 and 2 desulfate cell surface heparan sulfate proteoglycans (HSPGs) and modulate heparin-binding growth factor signaling in multiple cancers, including HCCs.

SULF1 downregulates HSPGs and suppresses heparin-binding growth factor signaling in HCC

- SULF1 is lost in the majority of HCC cell lines and approximately 30% of primary HCCs.
- SULF1 inhibits HCC tumor cell growth *in vitro* and in nude mice *in vivo*, partially through effects on gene expression mediated through histone H4 acetylation.

SULF2 has an oncogenic function opposite the tumor suppressor effect of SULF1 in HCC

- In contrast to the tumor suppressor effect of SULF1, SULF2 is up regulated in almost all HCC cell lines and in 60% of primary HCCs.
- Expression of SULF2 activates MAPK and Akt pathways, promotes HCC cell growth *in vitro* and *in vivo*, and shortens survival of HCC patients.
- Targeting SULF2 or the interaction between SULF2 and SULF1 may lead to novel therapeutics for the treatment of HCCs.

Conclusions

- Modulation of growth and survival signaling of heparin-binding growth factors and cytokines by the heparin-degrading endosulfatases SULF1 and SULF2 represents a novel paradigm in control of cell proliferation and apoptosis.
- The precise mechanisms by which SULF1 and SULF2 achieve their opposing effects on tumorigenesis remains to be elucidated.
- SULF1 and SULF2 represent potential new targets for the prevention and therapy of HCC.

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*Of interest

**Of considerable interest

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Figure 1. SULF1 and SULF2 Structure. Indicated from N-terminal (left) to C-terminal (right) are signal sequences (blue), conserved sulfatase domains (yellow) with catalytic C-formylglycines (pink line) followed by carboxy-terminal domains unique to SULFs (green) containing several subdomains: region (red) non-homologous to other sulfatase structures, polypeptide (purple) with little sequence similarity between SULF1 and SULF2, two predicted coiled-coils (pink), region with significant homology to glucosamine 6-sulfatase and n-acetylglucosaminyltransferase (yellow) with predicted secondary structure (blue) matching that following the catalytic core in sulfatase crystal structures, and regions of concentrated basic charge (light blue) that fit the heparin-binding motif BBXB (where B is K or R and X any amino acid) that appear important for binding cell surfaces and enzyme activity.

Figure 2.

A. Expression of SULF1 and SULF2 mRNA was determined in 11 HCC cell lines and normal human hepatocytes using RT-PCR. GAPDH was used to control for loading. B. Fluorescent In Situ Hybridization (FISH) analysis of gene copy number of the SULF1 gene locus in two SULF1 mRNA negative cell lines SNU387 and SNU449, and two SULF1 mRNA positive HCC cell lines SNU182 and SNU475.

Figure 3. Microarray analysis of gene expression showing that SULF2 mRNA expression is increased in approximately 60% of 139 HCCs.

Figure 4. Immunohistochemistry for SULF2 protein in benign liver tissue with low SULF2 mRNA expression (left panel), an HCC with high SULF2 mRNA (middle panel) and an HCC with low SULF2 mRNA (right panel). Nuclei were counterstained with hematoxylin.

Figure 5. Kaplan-Meier plots showing that high SULF2 expression is associated with decreased survival of patients with HCC.

Figure 6. Proposed hypothesis that SULF1 desulfates HSPGs leading to decreased growth factor signaling, while SULF2 increases GPC3, releases growth factor ligands from the storage sites on heparan sulfate, and increases growth factor binding to growth factor receptors at the HCC cell surface.