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SULF2, a heparan sulfate endosulfatase, is present in the blood of healthy individuals and increases in cirrhosis

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Abstract

Background—SULF2 is an extracellular sulfatase that acts on heparan sulfate proteoglycans and modulates multiple signaling pathways. It is normally bound to the cell surface but can be released into the medium of cultured cells. *SULF2* is known to be increased in cirrhotic liver compared to healthy liver. We asked whether SULF2 protein was present in the blood of healthy controls and increased in patients with liver cirrhosis.

Methods—We devised a sandwich ELISA for SULF2 using 2 novel monoclonal antibodies (mAbs) and measured its levels in sera of normal individuals and cirrhosis patients.

Results—SULF2 was higher in cirrhosis patients (1460 ± 1160 pg/ml, N =34) than healthy individuals (728 ± 400 pg/ml, N =37). SULF2 levels increased with age in both healthy and patient groups.

Conclusions—SULF2 may be a useful serologic biomarker for liver cirrhosis.

Keywords

SULF2; extracellular sulfatase; heparan sulfate; serum; ELISA; biomarker; cirrhosis

1. Introduction

The SULFs (SULF1 and SULF2) are members of the sulfatase family, which is comprised of 17 enzymes in human [1]. Its most studied members are the lysosomal enzymes, which degrade various sulfated substrates in the acidic milieu of the lysosome. Distinct from the

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lysosomal enzymes, the SULFs are extracellular enzymes that function optimally at neutral rather than acidic pH. The biological substrates for the SULFs are heparan sulfate (HS) chains associated with heparan sulfate proteoglycans (HSPGs) on the outside of cells. Whereas the lysosomal enzymes are exosulfatases, the SULFs are endosulfatases that remove internal 6-*O*-sulfate (6OS) within highly sulfated subdomains of HS [2,3]. Using specific sulfation patterns on their chains as recognition elements, HSPGs participate in a myriad of functions[4,5] as: 1) co-receptors for signaling receptors; 2) sequestration sites for signaling molecules (e.g., morphogens, cytokines, and growth factors); 3) ligands for cell adhesion; and 4) endocytic receptors. By virtue of the ability of the SULFs to edit the 6OS status of intact HS chains on the outside of cells, these enzymes modulate many signaling pathways [5]. In some cases the SULFs promote signaling (β-Catenin/Wnt, GDNF, PDGF) by releasing ligands from HSPG sequestration but in other contexts inhibit signaling (e.g., FGF-2) presumably by disrupting signaling complexes. The SULFs have been implicated as modulators of various developmental processes, regeneration and repair of injury, maintenance of stem cell populations, neuronal plasticity, and lipoprotein homeostasis [6–11]. SULF2, the focus of the present study, shows elevated expression in many human tumors and has been directly implicated in promotion of carcinogenesis in several cancers [12–14].

SULF1 and SULF2 are each synthesized as a preproprotein (871/870 aa in human) with a cleavable signal peptide, an amino-terminal region containing the catalytic site, a hydrophilic domain (HD), and a C-terminal region [3, 15]. After removal of the signal peptide, the proprotein (125 kDa) is cleaved within the HD domain by furin-related proteinases into a 75 kDa amino-terminal fragment, and a 50 kDa C-terminal fragment [16]. The 75 kDa and 50 kDa subunits are joined by disulfide bonds to form a heterodimer. The heterodimer as well as the 125 kDa proprotein both exhibit endosulfatase activity against heparan sulfate [16]. The SULFs associate with the cell surface by ionic interactions and can be released by a high salt wash [16–18]. For SULF1 (and presumably SULF2), the cell surface interaction is mediated by the HD via binding to heparan sulfate chains [18]. The HD is obligatory for heparan sulfate endosulfatase activity, by mediating the oriented presentation of HS chains to the catalytic site of the enzyme in a processive manner [19]. In SULF1 and SULF2 transfected cells, fully processed and enzymatically active forms are present in the conditioned medium, as well as on the cell surface [3,16,18]. The release of SULF2 into conditioned medium has also been documented for cancer cell lines [20–22]. The potential for SULF2 release from cells, together with the expression of *SULF2* transcripts in various normal tissues, prompted us to determine whether SULF2 is detectable in normal blood. In the present study, we establish its presence in plasma and serum by a novel sandwich ELISA. We apply this assay to demonstrate an increase of SULF2 in the serum of individuals with cirrhosis.

2. Materials and methods

2.1 Subjects and processing of blood

For the initial investigation to determine whether SULF2 was present in blood, healthy adults donated samples. The blood samples were collected under a UCSF Committee on

Human Research protocol. Blood was collected for serum or plasma in SST tubes or K2 EDTA-containing tubes (BD Vacutainer), respectively. Serum or plasma was processed according to the manufacturer's instructions, frozen and stored at −80°C. For comparisons between healthy controls and cirrhotis patients, the participants were enrolled under protocols approved by the Georgetown University Institutional Review Board between 2003 and 2010. Healthy individuals were visitors to Georgetown University Hospital accompanying patients coming for treatment or routine checkups. Cirrhotis patients were enrolled in collaboration with the Department of Hepatology and Liver Transplantation, Georgetown University Hospital, Washington, D.C. The diagnosis of cirrhosis was made by the attending physician based on clinical evaluation and/or liver biopsy. Cirrhotis patients had either chronic hepatitis C virus (HCV) infection or alcohol abuse as the primary diagnosis. Healthy and cirrhotis participants were matched on age. The cirrhotis patients were also matched on MELD score (degree of liver damage) between HCV and alcohol primary diagnosis groups. All participants donated a tube of blood collected according to the approved protocol in BD Vacutainer Serum Blood Collection Tubes. Serum was isolated within 6 hours of blood collection, aliquoted, and stored at −80°C until evaluation. All assays unless otherwise indicated were performed on second thaw. Basic characteristics of the study participants are summarized in Table 2.

2.2 Production and evaluation of SULF2 mAbs

Three mAbs (5D5, 8G1, and 5C12) were produced by immunizing *sulf2* null mice with recombinant human SULF2, as previously described [20–22]. The SULF1 goat anti-peptide antibody (G1.6) was previously described [10]. The specificity of the novel mAbs was evaluated by ELISA aqs follows. Immulon 2HB 96-well plates (Thermo Scientific) were coated with 100 ng per well heparin-BSA [23]. Recombinant human SULF1 and SULF2 were obtained by transfecting HEK293T cells with full-length cDNAs and collecting serum-free conditioned medium on day 3 [16]. ELISA wells were reacted with a two-fold dilution series of conditioned medium and incubated for 60 min at room temperature (RT). The plates were washed with PBS (Dulbecco's cation-free) containing 0.1% Tween 20 and then reacted with 3 μg/ml 5D5, 8G1, 5C12, or G1.6 for 60 min at RT. After washing, the mouse mAbs were detected with 1:6000 dilution of HRP-goat anti-mouse $IgG_{(H+L)}$(Jackson ImmunoResearch, West Grove, PA), the goat antibody was detected with a 1:6000 dilution HRP-swine anti-goat IgG (Invitrogen, Life Technologies, Carlsbad, CA). Color was developed with 1-Step® Ultra TMB-ELISA (Thermo Scientific). After terminating the reaction by the addition of 0.2M H_2SO_4, the absorbance at 450 nm was read on a Model 680 microplate reader (Bio-Rad Labs.). For immunoblotting, purified recombinant human SULF1, SULF2, or serum-free conditioned medium (CM) from MCF7 breast cancer cells [24] was separated by SDS-PAGE on 4–15% gradient TGX gels (Bio-Rad) and transferred onto a Problott PVDF membrane (Life Technologies). Immunoblotting was with the indicated mouse mAb (2 μg/ml), in conjunction with HRP-goat anti-mouse $IgG_{(H+L)}$ (Jackson ImmunoResearch), with ECL Plus (Thermo Pierce) for detection.

2.3 Sandwich ELISA

8G1 was biotinylated with the EZ-Link NHS-PEO_4-Biotinylation Kit (Thermo Pierce) according to the manufacturer's instructions. This served as the detection antibody. To

quantify SULF2 in MCF7 serum-free conditioned medium or human serum, wells of the 96-well plate (above) were coated with 0.5 μg of 5C12 mAb in 100 μl PBS overnight at 4°C. Control wells were coated with mouse IgG_1 as the isotype control (Affymetrix eBioscience). All remaining steps were performed at RT and all washes, except the last, were performed with three cycles of PBS containing 0.1% Tween 20 in a Bio-Rad ImmunoWash Model 1575 plate washer. After washing, non-specific binding sites were blocked by the addition of 200 μl per well of 3% BSA in PBS for 1 h. The material to be captured (dilutions of conditioned medium or serum) was made up to 100 μl/well with 1% BSA in PBS and preincubated for 30 min with 25 μg/ml polyclonal mouse IgG (Sigma-Aldrich, St. Louis, MO) in a parallel plate. The contents of the parallel plate were then transferred to the coated plate for 60 min capture. The wells were washed and incubated for 60 min with 100 μl of biotinylated 8G1 (2 μg/ml) in 1% BSA in PBS containing 25 μg/ml polyclonal mouse IgG. After washing, 10 ng of streptavidin-HRP (Jackson ImmunoResearch) in 100 μl 1% BSA in PBS with 0.05% Tween 20 was added to the wells for 30 min. After washing (one cycle every 5 min over 30 min), color was developed with the TMB substrate as above. Substrate reaction time was 15–30 min before quenching with 0.2 M H_2SO_4. A standard for the quantification of SULF2 was based on MCF7 CM. The absolute amount of SULF2 in MCF7 CM (amount of 75 kDa subunit) was quantified by quantitative immunoblotting with purified recombinant SULF2 [16] as the standard using 5D5 for detection, as described above. The advantage of using MCF7 CM as the standard was its relative stability. SULF2 in this form was stable for about 1 month when stored at 4°C in OptiMEM I (Life Technologies) with 0.1% sodium azide or indefinitely when stored in small aliquots at −80°C. Two different batches of MCF7 CM were used in the present study, one containing 616 ng/ml and the other 210 ng/ml SULF2.

For SULF2 determinations in serum, the assays were performed with 20 μl of serum per well. Three separate dilutions were prepared for each serum sample with triplicate determinations for each dilution. Background values were determined with $mIgG_1$ substituting for 5C12 as the capture reagent. The mean background value was subtracted from that with 5C12 to yield the specific signal, which was used to compute SULF2 by comparison with the MCF7 CM standard run on the same plate. Serum samples that had backgrounds greater than twice the intrinsic background ($mIgG_1$ capture signal with no serum added) or yielded a negative value were excluded.

2.4 Preclearing and immunoprecipitation

To deplete SULF2 from MCF7 CM, 220 μl CM was reacted for 150 min at 4°C with 10 μl of packed protein G-agarose (GE Healthcare) coupled with 8G1, 5D5 or mouse IgG1 (100 μg). To deplete SULF2 from serum, 435 μl of 50% serum in PBS was reacted for 150 min at 4°C with 10 μl of protein G-agarose coupled with 50 μg mAb. The ELISA was then used to quantify the amount of remaining SULF2 in the supernatants. SULF2 was immunoprecipitated from “characterized” fetal bovine serum (Hyclone, GE Healthcare) as follows. 50% serum in PBS was precleared of IgG by 2 sequential reactions with protein G-agarose. The supernatant was reacted overnight at 4°C with 20 μl of protein G-agarose coupled with 15 μg 8G1 or IgG_1. The beads were processed with sample buffer and subjected to SDS-PAGE and immunoblotting (5D5) as above.

2.5 Statistical analysis

Data are expressed as mean ± SD. The serum concentration of SULF2 for healthy controls and cirrhotis patients were not normally distributed. Therefore, the Mann-Whitney U test, a non-parametric test, was used to analyze differences. The tests were 2-tailed with a $p < 0.05$ taken as significant. Correlations between groups were determined with the Spearman's rank correlation test for non-normally distributed values and the Pearson's test for normally distributed values. One high outlier value was excluded from the healthy group and one from the cirrhosis group based on the Grubbs test ($p < 0.01$). GraphPad Prism version 5 (GraphPad Software) was used for statistical analysis.

3. Results

Development of an ELISA for SULF2

SULF2 is one of 1929 proteins that were identified in the human plasma proteome by mass spectrometry analysis of tryptic peptides [25]. The protocol was estimated to have a false discovery rate of 1%. To confirm the presence of SULF2 in blood and to quantify its level, we developed a sandwich ELISA employing a pair of SULF2 mAbs: 5C12 for capture and biotinylated 8G1 for detection. The antibodies recognized recombinant human SULF2 that was immobilized on heparin-coated plastic wells or after SDS-PAGE separation and electroblotting. (Fig. 1). The antibodies did not react with SULF1, yet a SULF1 specific antibody (G1.6) did (Fig. 1). We used serum-free conditioned medium (CM) from the MCF7 breast cancer cell line as the source of secreted SULF2 [24]. Dilutions of MCF7 CM were reacted with microtiter plastic wells coated with 5C12. The captured SULF2 was detected with biotinylated-8G1 with color generation (450 nM) by the addition of HRP-streptavidin and HRP substrate TMB (3,3′,5,5′-tetramethylbenzidine). We quantified the amount of SULF2 in MCF7 CM by quantitative immunoblotting (not shown) employing 5D5 mAb, another SULF2-specific mAb (Fig. 1), with purified recombinant SULF2 [16] as the standard for the 75 kDa subunit. We established a linear relationship between the ELISA signal and dilution of MCF7 CM over a range of 6 to 400 pg SULF2 per well (Fig. 2A). The background signal was determined by substituting a class-matched irrelevant mouse IgG_1 as the capture reagent for 5C12. The background signal was constant over the entire dilution range of MCF7. To verify that SULF2 in the MCF7 CM was responsible for the positive signal in the ELISA, we performed a preclearing experiment with two SULF2 mAbs (8G1 and 5D5). As shown in Fig. 2B, pre-incubation of MCF7 CM with protein G-agarose beads conjugated to either 8G1 or 5D5 reduced the ELISA signal to background, whereas control $mIgG_1$-coupled beads had no effect. We also established that heparin-agarose beads eliminated the ELISA signal from MCF7 CM (not shown).

Detection of SULF2 in blood of healthy controls

We first applied the ELISA to plasma and serum samples from healthy individuals. We found that about half of the samples exhibited very high signals with both the control $mIgG_1$ and 5C12 capture reagents. We suspected that this background was due to the presence of human anti-mouse antibodies in blood (HAMA) [26]. Consistent with this, inclusion of polyclonal mouse IgG (25 μg/ml) in the assay eliminated or strongly attenuated the background. With this modification of the assay, there was a linear increase in the specific

signal with serum concentration for all samples (Fig. 3A). To verify that these signals reflected actual SULF2, we tested for preclearing with two SULF2 mAbs. Reaction of serum samples with either 5D5- or 8G1-agarose beads eliminated the specific signals whereas control $mIgG_1$-beads had no effect (Fig. 3B). We next addressed the issue of whether components in serum might affect SULF2 detection. We carried out "spike and recovery" experiments in which varying amounts (low, medium or high) of exogenous SULF2 were added to four different sera and the % recovery was measured with the ELISA. We observed a relatively minor degree of suppression that did not exceed 26% (Table 1). Having established linearity over a range from 0 to 50% serum, SULF2 specificity of the assay, and a relatively low degree of signal suppression in a serum milieu, we analyzed a series of 11 serum samples from healthy donors. The samples were aliquoted and frozen shortly after collection and stored at −80°C. The samples were assayed on two separate days with triplicate assays on each sample. The intra-experiment coefficients of variation were 4.5 ± 2.4% and 5.1 ± 1.5% for the two sets of determinations. We obtained the same mean SULF2 values (621 ± 333 and 623 ± 403 pg/ml) for the two determinations (Fig. 4A). Individual values were highly correlated between the 2 determinations (Pearson $r= 0.93$, $p< 0.0001$) (Fig. 4B). We compared SULF2 concentration in serum and plasma of healthy donors (13 and 8 individuals, respectively). We found statistically indistinguishable levels (means ± SD) of 729 ± 243 pg/ml for the sera and 804 ± 373 pg/ml for the plasmas. However, we observed that with repeated refreezing and thawing cycles, the serum samples maintained their level of SULF2 whereas the plasma samples showed a marked decline in SULF2 (Fig. 4C).

Biochemical verification of SULF2 in serum

We wanted to confirm the presence of SULF2 in serum by independent biochemical means. We chose fetal calf serum as a source because an ELISA comparison indicated that it contained a ≈4-fold higher level of SULF2 than human serum. We subjected 10 ml aliquots of fetal calf serum to immunoprecipitation by agarose beads conjugated to 8G1 mAb or $mIgG_1$. After SDS-PAGE fractionation of the 8G1 immunoprecipitate, immunoblotting with 5D5 revealed a 75 kDa band, which co-migrated with the MCF7 SULF2 subunit (Fig. 4D). This band was not present when $mIgG_1$ was used for immunoprecipitation.

SULF2 is increased in the serum of patients with liver cirrhosis

Tatrai et al. [27] detected *SULF2* mRNA in healthy human livers and found a 2.8 fold increase ($p <0.01$) in livers with non-malignant fibrotic disease. Consistent with this report, we mined the Wurmbach microarray dataset [28] and identified an increase in *SULF2* mRNA in cirrhotic (13) vs. healthy (10) livers (2.8–2.9 fold, $p <0.001$) (www.oncomine.org). Since secreted products of the liver have ready access to the blood across the discontinuous endothelium of liver sinusoids, we asked whether SULF2 was elevated in the blood of cirrhotis patients. The cirrhosis patients had a primary etiology of either alcoholic liver disease or HCV infection. The characteristics of the individuals investigated are presented in Table 2. There was a broad distribution of SULF2 concentration in each group (Fig. 5). We found a significant increase of SULF2 ($p= 0.001$) in the sera of cirrhosis patients (1460 ± 1160 pg/ml, N= 34, median=1050 pg/ml) vs. healthy individuals (728 ± 400 pg/ml, N =37, median=703 pg/ml). Of the patients with cirrhosis,

both alcoholic cirrhosis patients (1410 ± 1010 pg/ml, median=1010 pg/ml, N= 15) and HCV cirrhosis patients (1503 ± 1290 pg/ml, median=1100 pg/ml, N= 19) had similar elevations in SULF2 (p=0.0011 and 0.033, respectively) compared to healthy controls. The diagnostic value of serum SULF2 for differentiating cirrhotic individuals (alcoholic and HCV) from healthy controls was evaluated by the area under the curve (ROC) analysis. ROC was 0.727 with a 95% confidence interval of 0.606 to 0.849. At a cutoff value of >801 pg/ml of SULF2, the specificity and sensitivity of the assay were 67.7% and 70.3%, respectively.

Unexpectedly, serum SULF2 concentration increased with age in both healthy controls (Spearman r= 0.59, p= 0.0001) and cirrhosis patients (Spearman r=0.45, p= 0.0081) (Fig. 6A). When the median age (56 years) was used to subdivide the groups (Table 3), SULF2 was significantly higher in the older group in healthy controls (p=0.003) as well as HCV cirrhosis patients (p= 0.010 with a trend for this association in the alcoholic cirrhosis group (p= 0.14). Age could not account for the elevated SULF2 in cirrhosis patients since the age distribution of the healthy controls (mean ± SD= 54.3 ± 9.2; median=56) closely matched that of the cirrhosis patients (mean ± SD= 54.9 ± 7.2; median=55). There was no association of either gender or race (Caucasian vs. African-American) with SULF2 levels in either the cirrhosis patients or the healthy controls (Table 3). There was a statistically significant correlation (p= 0.047) between MELD score and SULF2 among the cirrhosis patients (Fig. 6B).

Discussion

We describe a sandwich ELISA which measures soluble SULF2 with a detection limit of ≈6 pg per well. We have used two novel SULF2 mAbs (8G1 and 5C12) for detection and capture and a third novel one (5D5) for immunoprecipitation and immunoblotting. Employing both immunoblotting and ELISA, we verified that these three mAbs react with SULF2 but not SULF1.

In addition to the potential use of the ELISA to measure blood SULF2, we demonstrate that the assay detects SULF2 in the serum-free conditioned medium of a human breast carcinoma cell line (MCF7). SULF2 secretion has been reported for a number of cancer cell lines and the ELISA may prove useful for the quantification of native or recombinant SULF2 in the media of cultured cells.

A global proteomics analysis of human plasma detected SULF2 as one of 1929 proteins that were judged to be present with a high level of confidence. Application of the ELISA in the present study confirms the presence of SULF2 in the blood of healthy individuals. The distribution of concentrations is broad with a mean of ≈700 pg/ml. Biochemically, we have established the presence of the 75 kDa amino-terminal subunit in serum by immunoblotting. This finding is consistent with the ELISA data, since the capture and detection antibodies are both reactive with this subunit. Interestingly, the SULF2 peptide found in the proteomics analysis of plasma [25] derives from the 50 kDa subunit, indicating that this subunit is also present in serum. Both subunits are required for endosulfatase activity against heparin/heparan sulfate as well as arylsulfatase activity against the pseudosubstrate 4-MUS [16]. The tissue sources of SULF2 in serum are unclear. Gene expression data in mouse demonstrates

sulf2 in many adult tissues (www.biogps.org). Among hematopoietic cells, prominent *sulf2* expression occurs in populations of monocytes, macrophages, dendritic cells and NK cells (www.immgen.org). We have established the expression of the SULF2 75 kDa subunit in human peripheral blood mononuclear cells by immunoblotting of total cell lysates (not shown). It should be noted that a proteomics cataloging of human urine tryptic peptides also detected SULF2 [29]. The presence of the protein in urine remains to be confirmed by application of the ELISA.

The upregulation of *SULF2* transcripts in human fibrotic liver led us to examine whether the protein was elevated in the serum of cirrhosis patients. Indeed we found an almost two-fold elevation in cirrhosis patients of both alcoholic and HCV etiology. Cirrhosis represents the final stage of progressive fibrosis in the liver and is the leading cause of liver-related morbidity and death [30]. Increasing fibrosis, culminating in cirrhosis, results in liver dysfunction at the level of individual cells and the whole organ [30]. Histology on biopsies is the currently accepted standard for assessing the extent of fibrosis in the liver [30]. Recently, there has been considerable interest in the identification of serum biomarkers for liver fibrosis, one important application being the staging of HBV and HCV infected patients by non-invasive tests [31, 32]. The advantages of a serum test over biopsy are cost, comfort of the patient, and the opportunity to follow patients longitudinally. A number of indices, which combine several parameters, perform well in discriminating the stages of liver fibrosis [31, 32]. The serum level of SULF2 with a correction for the age dependency may provide an independent marker that can be combined with already validated markers to facilitate the noninvasive staging of fibrosis.

It is noteworthy that SULF2 has been recently linked to another form of fibrosis in that *SULF2* transcripts are elevated in lung during idiopathic pulmonary fibrosis (IPF) [33]. SULF2 protein is localized to hyperplastic type II alveolar epithelial cells (AEC) where it regulates TGF-β signaling. TGF-β is pivotal to IPF by promoting the conversion of Type II AEC to myofibroblasts, the principle cellular source of fibrosis. Correspondingly, TGF-β is a major profibrogenic cytokine that acts on liver myofibroblasts during cirrhosis [30]. SULF2 thus deserves consideration, not only for its potential as a serum biomarker for cirrhosis but also for playing a mechanistic role in this disease.

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Abbreviations

4-MUS	4-methylumbelliferyl sulfate
6OS	6-*O*-sulfate
CM	conditioned medium
HAMA	human anti-mouse antibodies
HD	hydrophilic domain

HSPG	heparan sulfate proteoglycan
IPF	idiopathic pulmonary fibrosis
TMB	3,3′,5,5′-tetramethylbenzidine

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Highlights

- SULF2 is an extracellular sulfatase that acts on heparan sulfate proteoglycans and modulates multiple signaling pathways.
- SULF2 is normally bound to the cell surface but can be released into the medium of cultured cells.
- *SULF2* Transcripts encoding SULF2 qare known to be increased in cirrhotic liver compared to healthy liver.
- We sought to determine whether SULF2 protein was present in the blood of healthy controls and increased in patients with liver cirrhosis.
- We devised a sandwich ELISA for SULF2 using two novel monoclonal antibodies (mAbs) and measured its levels in sera of normal individuals and cirrhotic patients.
- SULF2 was higher in cirrhotic patients (1460 ± 1160 pg/ml, N =34) than healthy individuals (728 ± 400 pg/ml, N =37). SULF2 levels increased with age in both healthy and patient groups.
- SULF2 may be a useful serologic biomarker for liver cirrhosis.

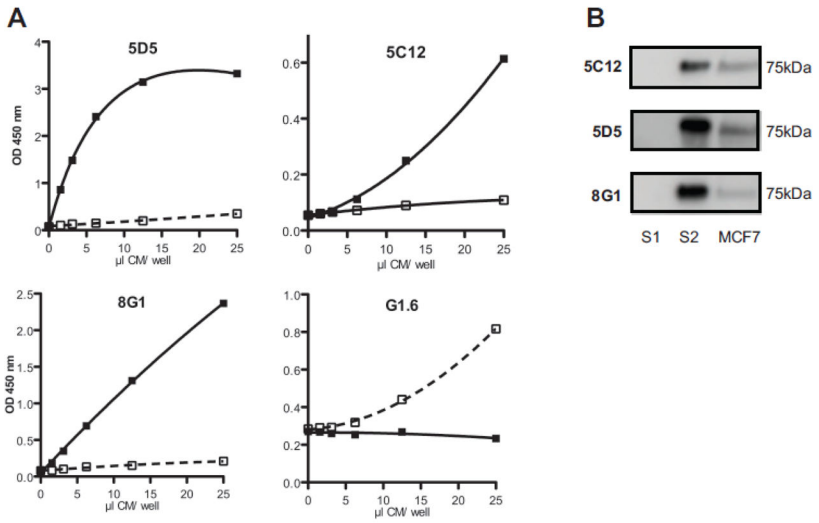

Fig. 1.

Specificity of SULF2 mAbs. A. Reactivity of 5D5, 5C12, 8G1, and G1.6 with varying dilutions of condition medium (CM) from SULF2- (■) or SULF1-transfected HEK293T cells (□). The CMs were applied to wells coated with heparin-BSA. B. Immunoblotting reactivity of 5C12, 5D5, and 8G1 on purified HSULF1 (S1) (20 ng), HSULF2 (S2)(20 ng) or MCF7 CM (30 µl).

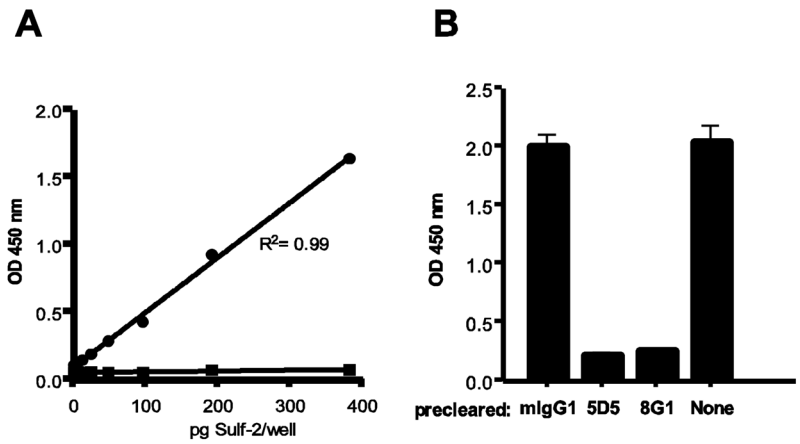

Fig. 2.

Detection of SULF2 in MCF CM. A. Application of the ELISA to MCF7 CM. The amount of SULF2 was determined by immunoblotting comparison with purified SULF2. ● denotes signal with 5C12 as capture antibody, and ■ denotes background signal with irrelevant $mIgG_1$ as capture reagent. Linear regression analysis was used to fit straight lines. R^2 indicates the goodness of fit. B. MCF7 CM was reacted with beads conjugated to irrelevant $mIgG_1$, 5D5 or 8G1, or was not treated (none). The supernatants were evaluated in the ELISA for remaining SULF2. Means ± SDs for three replicates are shown.

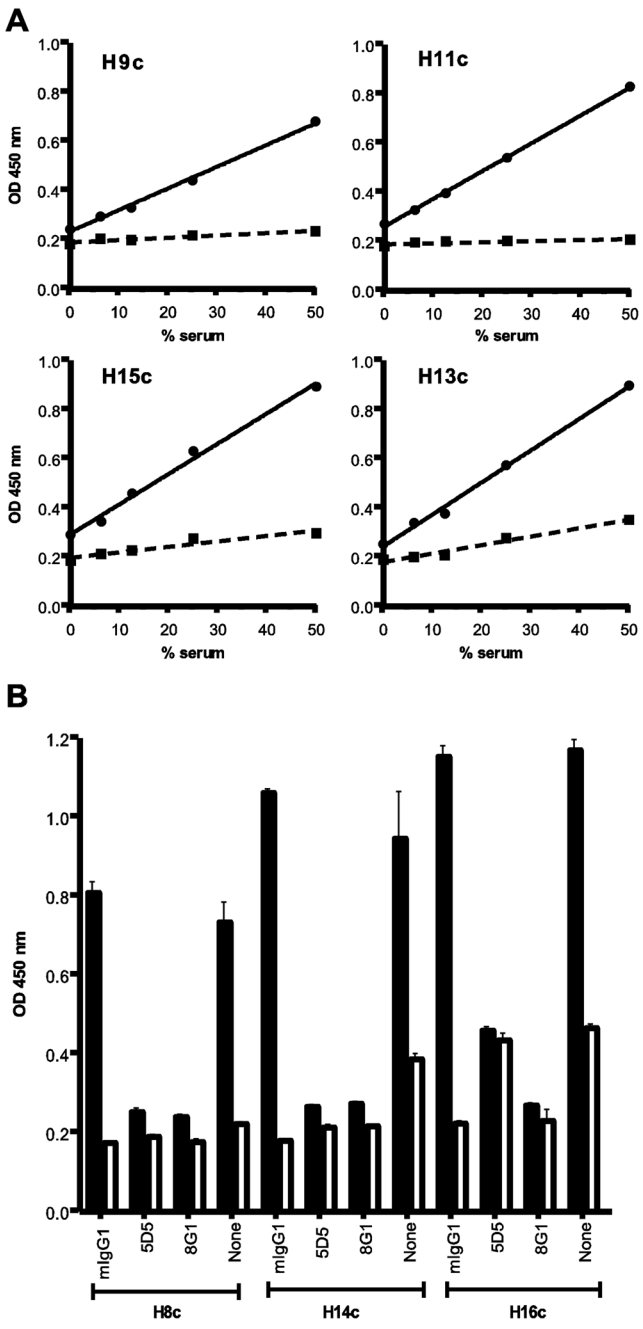

Fig. 3.

Testing of sera from four healthy controls by ELISA. A. ELISA signals with 5C12 (●) or $mIgG_1$ (■) for capture are shown as a function of serum dilution. There is no background with H9c and H11c but there is slight background ($mIgG_1$ signal with serum relative to $mIgG_1$ without serum for H15c and H13c. B. The indicated healthy control serum samples were reacted with beads conjugated to $mIgG_1$, 5D5 or 8G1, or not treated (none). The supernatants were tested in the ELISA. Solid bars denote signals with 5C12 for capture and open bars the signals with $mIgG_1$ for capture. The means ± SDs are based on 3 replicate determinations.

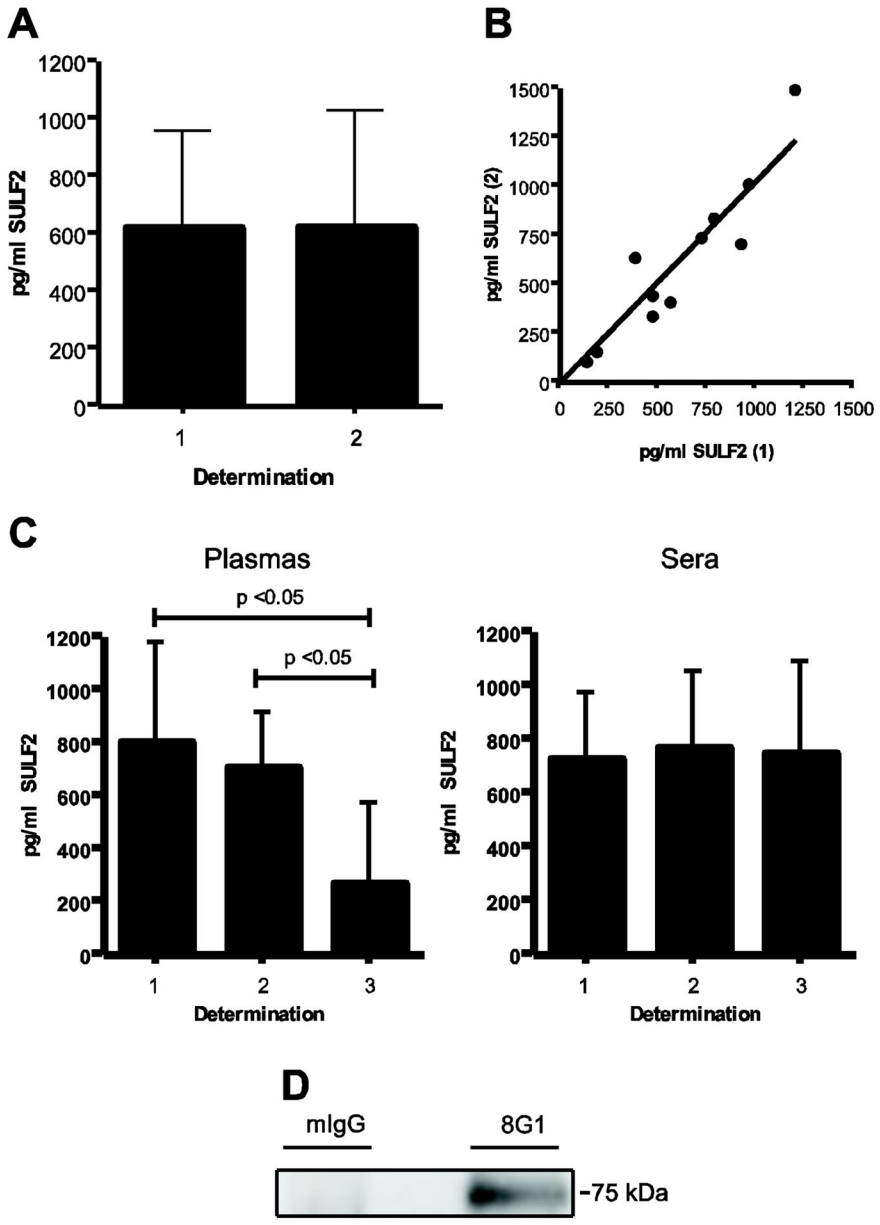

Fig. 4.

SULF2 determinations in serum and plasma. A. Serum samples from 11 healthy controls assayed on two different days. Means ± SDs are shown for the determinations. B. Correlation between determinations on the same samples. The straight line was fit by linear regression with a Pearson r of 0.93, $p < 0.0001$. C. 13 serum samples and 9 plasma samples from healthy controls were assayed for SULF2 repeatedly after freezing, storage (−80°C) and thawing cycles. Determination 1 was performed after the first thaw, determination 2 after refreezing and 28 days of storage, and determination 3 after refreezing and an additional 32 days of storage. Means±SDs are based on 3 replicate determinations. p values were determined by one-way ANOVA with Tukey's multiple comparisons test. D. Fetal bovine serum was immunoprecipitated with 8G1 or mIgG and immunoblotted with 5D5.

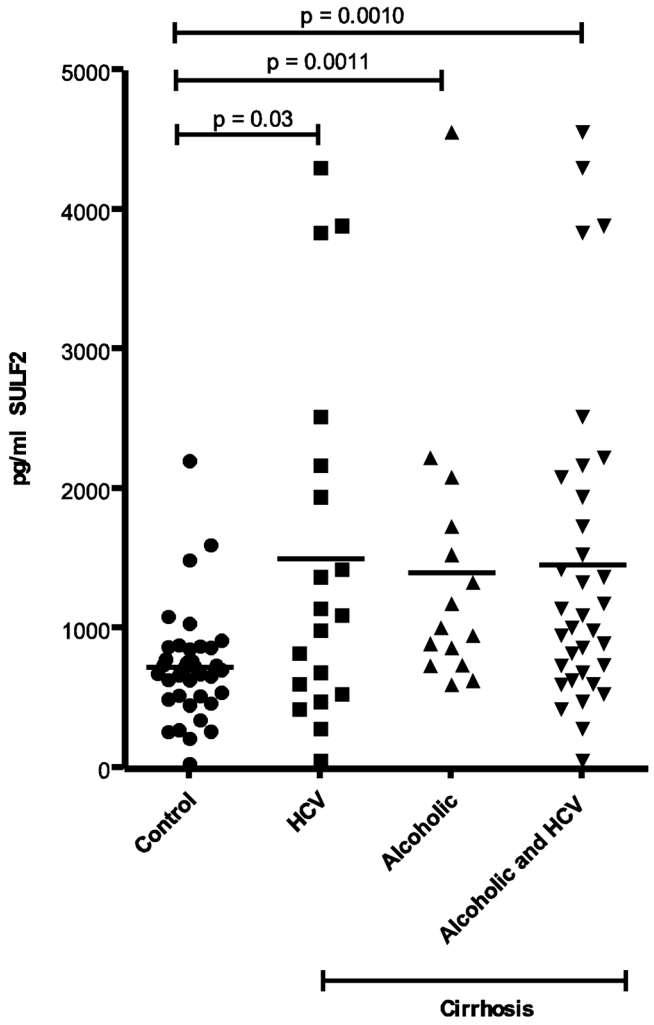

Fig. 5.

Serum SULF2 levels in healthy controls (N= 37) and cirrhosis patients. Cirrhosis patients are shown as separate groups of alcoholic cirrhotic (N= 15) and HCV infected cirrhotic (N= 19) and pooled together (N= 34). The statistical comparisons were performed with the Mann-Whitney test. One outlier value was eliminated from the control group and another from the alcoholic cirrhotic group by application of the Grubbs test with $p<0.01$.

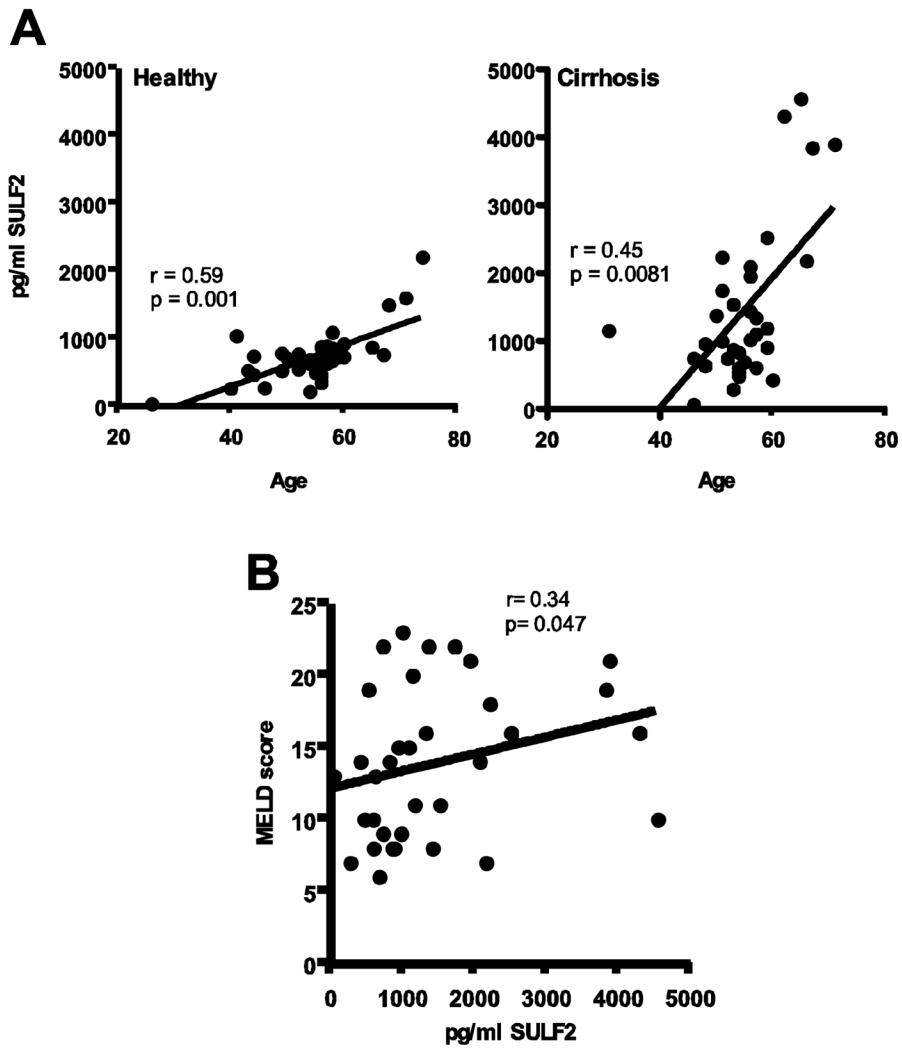

Fig. 6.

Correlation of serum SULF2 with age and MELD score. A. SULF2 vs. Age for healthy controls (left) and cirrhosis patients (right). Straight lines were fit by linear regression. Spearman r coefficients and p values are shown. B. MELD score vs. SULF2 for cirrhosis patients. Straight line was fit by linear regression. Spearman r coefficient and p value are shown.

Table 1

Percent recovery of exogenously added SULF in the presence of serum

Spike	Serum1	Serum2	Serum3	Serum4	Mean ± SD
Low	89.6	129.4	80.1	78.0	94.3 ± 24.0
Medium	77.5	70.5	78.0	68.8	73.7 ± 4.7
High	88.4	68.3	87.2	70.2	78.5 ± 10.7

Low (24.5 pg/well), medium (71.9 pg/well), and high (171 pg/well) amounts of SULF2 in MCF CM were spiked into 50% serum samples or into the standard sample diluent alone, and these were assayed by the ELISA. 3 independent samples were analyzed for each condition with triplicate determinations on each sample. The amt of exogenously added SULF2 was quantified without serum and the percent recovery of this amount was determined in the presence of serum.

Table 2

Characteristics of subjects

Feature	Healthy	Alcoholic	HCV
No. Subjects	37	15	19
Age	54.3 ± 9.2 (26 – 74)	53.9 ± 5.0 (46 – 65)	55.7 ± 8.5 (31 – 71)
Sex (M/F)	25/9	9/6	14/5
Race (C/A/H)	24/7/1	11/2/2	11/7/1

C, A, and H denote Caucasian, African-American, and Hispanic, respectively.

Table 3

Relationships between serum SULF2 levels and other subject characteristics

Group	Characteristic	Gender	No.	Serum SULF2 (pg/ml)	p value
Healthy	Gender	Male	25	745 ± 439	NS
		Female	9	755 ± 331	
	Race	Caucasian	25	745 ± 474	NS
		African-Am	7	730 ± 137	
	Age	<56 yrs	17	548 ± 249	0.003
		≥56 yrs	19	913 ± 433	
HCV	Gender	Male	14	1740 ± 1420	NS
		Female	5	842 ± 427	
	Race	Caucasian	11	1412 ± 1400	NS
		African-Am	7	1720 ± 1280	
	Age	<56 yrs	9	706 ± 421	0.010
		≥56 yrs	10	2220 ± 1400	
Alcoholic	Gender	Male	9	1110 ± 371	NS
		Female	6	1850 ± 1500	
	Race	Caucasian	11	1440 ± 1180	ND
		African-Am	2	1270 ± 369	
	Age	<56 yrs	9	1110 ± 577	NS
		≥56	6	1840 ± 1390	

The p values were determined by the Mann-Whitney test. ND, not done (too few values for the statistical test)