Cellular Interaction and Cytotoxicity of the Iowa Mutation of Apolipoprotein A-I \((\text{ApoA-I}_{\text{iowa}})\) Amyloid Mediated by Sulfate Moieties of Heparan Sulfate*

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The single amino acid mutation G26R in human apolipoprotein A-I \((\text{apoA-I})\) is associated with familial amyloid polyneuropathy III. ApoA-I carrying this mutation \((\text{apoA-I}_{\text{iowa}})\) forms amyloid fibrils in vitro. Heparan sulfate \((\text{HS})\) is a glycosaminoglycan that is abundant at the cell surface and in the extracellular matrix. Although HS and its highly sulfated domains are involved in aggregation of amyloid-\(\beta\) and accumulate in cerebral amyloid plaques of patients with Alzheimer disease and mouse models of this disease, the role of HS in familial amyloid polyneuropathy III has never been addressed. Here, we used cell models to investigate the possible role of HS in the cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid. Wild-type CHO cells, but not pgsD-677 cells, an HS-deficient CHO mutant, demonstrated uptake of \(\text{apoA-I}_{\text{iowa}}\) amyloid after incubation with the amyloid. Addition of sulfated glycosaminoglycans to culture media prevented interaction with and cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid to CHO cells. Elimination of cell surface HS or inhibition of HS sulfation with chemical reagents interfered with interaction of \(\text{apoA-I}_{\text{iowa}}\) amyloid with CHO cells. We also found that cellular interaction and cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid were significantly attenuated in CHO cells that stably expressed the human extracellular endoglucomasine 6-sulfatases \(\text{H}5\text{ulf-I}\) and \(\text{HSulf-2}\). Our results thus suggest that cell surface HS mediates cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid and that enzymatic remodeling of HS mitigates the cytotoxicity.

Sulfate moieties of cell surface HS are critical for mediating apoA-I amyloid cytotoxicity. The G26R apolipoprotein A-I \((\text{apoA-I}_{\text{iowa}})\) mutation causes familial amyloid polyneuropathy.

**Results:** ApoA-I \((\text{apoA-I}_{\text{iowa}})\) amyloid cellular interaction and cytotoxicity depended on cell surface heparan sulfate (HS). Enzymatic remodeling of HS by extracellular sulfatase mitigated cytotoxicity.

**Conclusion:** Sulfate moieties of cell surface HS are critical for mediating apoA-I amyloid cytotoxicity.

**Significance:** Enzymatic remodeling of HS may be a novel concept for regulating actions of amyloid on cells.

Apolipoprotein A-I \((\text{apoA-I})\) is the major protein constituent of human high density lipoproteins, which transport excess cellular cholesterol through the plasma compartment to the liver \((1, 2)\). ApoA-I contains 11/22-mer tandem repeats with a high tendency to form amphipathic \(\alpha\)-helices that can bind to lipid surfaces \((3)\). Approximately 50% of the secondary apoA-I structure consists of \(\alpha\)-helices, and the N terminus forms an \(\alpha\)-helix bundle \((4, 5)\). In the hereditary amyloidosis known as familial amyloid polyneuropathy, some apoA-I mutants form amyloid fibrils. These apoA-I mutations facilitate the proteolysis of apoA-I, and the resulting N-terminal fragments deposit as fibrils in different organs, including the kidney, liver, and heart \((6–9)\). The first apoA-I mutation discovered that was associated with familial amyloid polyneuropathy occurred in an Iowa pedigree \((10)\) and was identified as a G26R single substitution mutation \((11, 12)\). As with most amyloidogenic apoA-I mutations, the N-terminal 1–83-residue fragment of the Iowa apoA-I variant is deposited predominantly as amyloid fibrils \((11)\). This substitution destabilizes the N-terminal \(\alpha\)-helix bundle and increases formation of amyloid fibrils of the N-terminal fragment \((13)\).

Heparan sulfate \((\text{HS})\) is a sulfated polysaccharide comprising repeating units of glucosamine and glucuronic or iduronic acid. HS is a member of the glycosaminoglycan \((\text{GAG})\) family. One or more HSs bind covalently to a core protein to form heparan sulfate proteoglycan \((\text{HSPG})\), which exists ubiquitously at cell surfaces and in the extracellular matrix \((14)\). The structure of HS varies. HS includes domains that are highly sulfated and partially sulfated or not sulfated \((15)\). Heparin is a structural protein constituent of mammalian cell surfaces and in the extracellular matrix. Although HS and its highly sulfated domains are involved in aggregation of amyloid-\(\beta\) and accumulate in cerebral amyloid plaques of patients with Alzheimer disease and mouse models of this disease, the role of HS in familial amyloid polyneuropathy III has never been addressed. Here, we used cell models to investigate the possible role of HS in the cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid. Wild-type CHO cells, but not pgsD-677 cells, an HS-deficient CHO mutant, demonstrated uptake of \(\text{apoA-I}_{\text{iowa}}\) amyloid after incubation with the amyloid. Addition of sulfated glycosaminoglycans to culture media prevented interaction with and cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid to CHO cells. Elimination of cell surface HS or inhibition of HS sulfation with chemical reagents interfered with interaction of \(\text{apoA-I}_{\text{iowa}}\) amyloid with CHO cells. We also found that cellular interaction and cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid were significantly attenuated in CHO cells that stably expressed the human extracellular endoglucomasine 6-sulfatases \(\text{H}5\text{ulf-I}\) and \(\text{HSulf-2}\). Our results thus suggest that cell surface HS mediates cytotoxicity of \(\text{apoA-I}_{\text{iowa}}\) amyloid and that enzymatic remodeling of HS mitigates the cytotoxicity.

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‡ The abbreviations used are: apoA-I, apolipoprotein A-I; HS, heparan sulfate; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; A\(\beta\), amyloid \(\beta\); AD, Alzheimer’s disease; \(\text{apoA-I}_{\text{iowa}}\), amyloid carrying the G26R mutation; MTI, 3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TMRE, tetramethylrhodamine, ethyl ester; HA, hyaluronic acid.
analogue of HS that consists mostly of highly sulfated domains. The highly sulfated structure in HS is believed to be an essential feature in molecular interactions between HS and many protein ligands (16). Extracellular endosulfatases, Sulf-1 and Sulf-2, have removed C-6 sulfate groups in highly sulfated domains of HS and regulated protein-ligand interactions with HS (17–20).

In the first study that linked GAGs and amyloidosis, Snow and Kisilevsky (21) found that GAGs were associated with tissue amyloid, which suggested that HS may be involved in the process of amyloid A (AA) amyloidosis. HS was also associated with various types of amyloid in disorders other than AA amyloidosis, including the amyloid in amyloid light chain amyloidosis (22), amyloid-β (Aβ) of Alzheimer disease (AD) (23–26), prion protein in Creutzfeldt-Jakob disease (27), and islet amyloid polypeptide in the islets of Langerhans of type 2 diabetes (28). We and others showed that HS mediated the cellular uptake of Aβ and the cellular association with Aβ (29, 30). With regard to apoA-I amyloidosis, Ramella et al. (31) reported that apoA-I interacted with heparin at acidic pH. However, involvement of HS in the pathological processes of apoA-I amyloidosis remains to be elucidated.

The goal of this study was to understand the involvement of HS in the pathogenesis of apoA-I amyloidosis. By using CHO cells and an HS-deficient CHO variant, we showed that the cytotoxicity of amyloid consisting of G26R apoA-I (1–83) fragment (apoA-I\textsubscript{Iowa} amyloid) was mediated by cell surface HS through cellular interaction of apoA-I\textsubscript{Iowa} amyloid. Cytotoxicity and cellular interaction of apoA-I\textsubscript{Iowa} amyloid were suppressed in the presence of an excess amount of sulfated GAGs and were substantially attenuated by expression of human Sulf-1 (HSulf-1) and Sulf-2 (HSulf-2). Therefore, enzymatic remodeling of cell surface HS may be one approach for modulating the cytotoxicity of apoA-I\textsubscript{Iowa} amyloid.

**Experimental Procedures**

**Materials**—Porcine intestinal mucosa heparin (average molecular weight 13,000 and <38% sulfur content) and porcine intestinal mucosa HS (average molecular weight of 13,655, and sulfur content of 5.51%) were purchased from Celsus Laboratories (Cincinnati, OH). Hyaluronic acid from human umbilical cords (molecular weight of 50,000–800,000) was purchased from MP Biomedicals (Santa Ana, CA). An anti-actin antibody was purchased from Sigma. CHO cells that were stably transfected with cDNA encoding HSulf-1 or HSulf-2 were established as described previously (32). FITC-labeled heparin was purchased from Polysciences, Inc. (Warrington, PA). OptiMEM reduced-serum medium was purchased from Thermo Fisher Scientific (Waltham, MA). Porcine intestinal mucosa heparin was coupled, via its reducing end, to BSA based on the procedure of Najjam et al. (33). The conjugates were used as “heparin-BSA.”

**Preparation of ApoA-I Proteins**—cDNA encoding N-terminal fragment 1–83 of apoA-I was acquired by using PCR methods with full-length human apoA-I cDNA as the template. The human apoA-I mutation to obtain the G26R variant was produced by means of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The pET32a+ expression vector (Novagen, Madison, WI) was used for cDNA ligation. The pET32a+ vector contains an ampicillin resistance gene for clonal selection and a His tag at the fusion linker location so that the expressed protein can be purified on a nickel affinity column. A site recognizing thrombin was established at the fusion junction so that cleavage of the thioredoxin by thrombin would produce a protein with the N terminus containing the two extra amino acids Gly-Ser (34). The construct was transformed into Escherichia coli strain BL21 Star (DE3) (Thermo Fisher Scientific, Waltham, MA). ApoA-I fusion proteins were expressed and purified as described before (5, 35). The apoA-I preparations demonstrated at least 95% purity, as found by SDS-PAGE followed by Coomassie Brilliant Blue staining. ApoA-I variants were freshly dialyzed from 6 M guanidine hydrochloride solution into PBS before use in all experiments.

**Preparation of ApoA-I Amyloid Fibrils**—The solution containing the N-terminal fragment of apoA-I with or without the Iowa mutation was diluted with PBS to give a final concentration of 0.3 mg/mL, and it was then incubated in a microcentrifugation tube in a rotating mixer for 7 days at 37 °C to form amyloid fibrils.

**Production of Monoclonal Anti-apoA-I Antibodies**—Anti-apoA-I antibodies were prepared as described previously (36). Briefly, a female BALB/c mouse (8 weeks of age; Japan SLC, Hamamatsu, Japan) was immunized biweekly (a total of four times) with wild-type apoA-I. Antigen (50 μg) was injected subcutaneously at multiple sites on the back in the form of an emulsion of Freund’s complete (for primary immunization) or incomplete adjuvant (for booster immunizations) (BD Biosciences) and sterile saline (1:1; 0.2 ml). The mouse received intraperitoneal and intrasplenic injections of the antigen (50 μg each) dissolved in sterile saline (0.5 and 0.2 ml, respectively). After 3 days, splenocytes (1.3 × 10⁸ cells) from the mouse were fused with P3/NS1/Ag4-1 myeloma cells (2.6 × 10⁷ cells) by using 40% PEG 4000 in sterile PBS containing 10% (v/v) DMSO and a 0.001% poly-L-arginine-HCl solution (1 ml). The fused cells were cultured in hypoxanthine/aminopterin/thymidine medium supplemented with 10% BrriClone (Archport, Dublin, Ireland) under 5% CO₂, 95% air at 37 °C for ~10 days. Hybridomas secreting the anti-apoA-I antibodies were assessed via ELISA with microplates coated with wild-type apoA-I (conjugated with BSA), were expanded in HT medium, and were cloned by means of a limiting dilution. A monoclonal anti-apoA-I antibody, which was secreted in culture medium from one of these hybridoma clones (clone Wt20-7), was used in the experiments.

**Cell Culture**—Wild-type CHO cells and their variants were cultured in DMEM/F-12 medium (Sigma) supplemented with 10% heat-inactivated FBS (Lonza Group Ltd., Basel, Switzerland), 100 units/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) at 37 °C in an atmosphere containing 5% CO₂.

**Cytotoxicity Assay**—Cytotoxicity was determined by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (37). Wild-type CHO cells and mutant CHO cells were cultured as described above. Cells were plated on 24-well plates in DMEM/F-12 medium containing 10% FBS. After 12 h of culture, cells were treated with 1 μM apoA-I\textsubscript{Iowa} amyloid for 12 h at 37 °C. Cell viability was quantified by means of MTT reduction. MTT was added to each well at a final con-
Sulfate Moieties of HS Mediate Toxicity of ApoA-I Amyloid

centration of 0.5 mg/ml. After 1 h, the medium was removed, and the resulting formazan crystals were dissolved in DMSO. Absorbance values of formazan were determined at 570 nm, with subtracted absorbance at 650 nm, by using an Infinite M200 microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Analysis of Cellular Interaction of ApoA-Iowa Amyloid via Western Blotting and Dot Blotting—Cells were plated on 6-cm culture dishes and cultured for 12 h, after which they were incubated with 1–10 μM apoA-Iowa amyloid at 37 °C for 12 h to evaluate cellular interaction of the amyloid. To remove the cell surface–associated apoA-I fibrils, the cells were further treated with 0.25% trypsin/EDTA (Sigma) for 10 min at 37 °C. After incubation, whole cell lysates were prepared by using TCA precipitation (38). Briefly, cells were washed three times with PBS and were then treated with 10% TCA (w/v) in PBS. After incubation on ice for 30 min, samples were centrifuged at 1000 × g for 5 min at 4 °C. The resulting precipitates were dissol

Degradation Assay—Cells were plated and treated with 1 μM apoA-Iowa fibrils for 12 h. The cells were then washed with PBS three times and cultured for an additional 12 h in fresh DMEM/F-12 medium in the presence or absence of chloroquine (Wako Pure Chemical Industries). Whole cell lysates were prepared as described above, and the apoA-I content was analyzed by dot blotting.

Immunocytochemistry—Cells were plated on a poly-L-lysine-coated glass and cultured for 12 h. They were then incubated with apoA-Iowa amyloid (1 μM) at 37 °C for 12 h to investigate cellular interaction of the amyloid. In some experiments, cells were pretreated with β-xyloside (2.5 mM) or sodium chloride (100 mM) for 24 h. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After the cells were washed three times with PBS, they were blocked and permeabilized with 10% normal goat serum and 0.05% saponin in PBS at room temperature for 20 min. They were then incubated with anti-apoA-I antibody and an anti-EEA1 (early endosomal antigen 1) antibody (Abcam, Cambridge, UK) followed by an Alexa 568- and 488-conjugated secondary antibody (Thermo Fisher Scientific). Stained specimens were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA) and were examined with an A1R confocal laser microscope (Nikon Corp., Tokyo, Japan). For quantitative analysis of amyloid-positive cells, 5–10 fields were randomly selected, and the total number of cells and the number of amyloid-positive cells were counted. The experiment was repeated three times, and the mean ratio of amyloid-positive cells/total cells was calculated.

Detection of ApoA-Iowa Amyloid-Heparin Interaction—Interaction of apoA-Iowa amyloid with heparin was evaluated by using a nitrocellulose filter binding assay (39) with a slight modification. Briefly, 0.1 mg/ml apoA-Iowa amyloid was incubated alone or with FITC-labeled heparin (12.5 μg/ml) for 12 h at 37 °C, pH 7.4. Aliquots (2.5 μl of each sample) were applied to a nitrocellulose filter with a 0.45-μm pore size (Merck KGAA, Darmstadt, Germany), and the filter was rinsed with PBS. The fluorescent signals of complexes of FITC-labeled heparin and amyloid trapped by the filter were visualized by means of an LAS-4000 luminescent image analyzer (Fujifilm). After this visualization, apoA-Iowa amyloid on the filter was probed with the monoclonal anti-apoA-I antibody followed by HRP-labeled anti-mouse antibody and ImmunoStar LD. Signals were visualized by using an LAS-3000 luminescent image analyzer.

ELISAs for Evaluating Affinity of ApoA-Iowa Amyloid for Heparin—One hundred ng/ml heparin-BSA in PBS was added to each well (100 μl/well) of a 96-well plate (Immulon 2HB, Chantilly, VA), and the plate was placed at 4 °C overnight. The wells were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and then blocked with EzBlockChemi (ATTO Corp., Tokyo, Japan) at room temperature for 1 h. ApoA-I fibrils were added to each well (10 and 100 nM and 1, 5, 10, 20, and 50 μM in PBS) and placed at 37 °C for 12 h. Then the wells were washed three times and incubated with the anti-apoA-I antibody in EzBlockChemi (100 μl/well) at room temperature for 1 h. The wells were washed and incubated with an alkaline phosphatase-linked secondary antibody (Cell Signaling Technology, Inc.) at room temperature for 30 min. After washing as above, the wells were incubated with 1 mg/ml p-nitrophenyl phosphate (Thermo Fisher Scientific) in cacodylate buffer, pH 9.2, at room temperature for 20 min, and A405 nm was read on an Infinite M200 microplate reader. The binding affinities of apoA-I fibrils for heparin (Kd values) were calculated by using the Hill plot as shown in Equation 1,

\[
Y = B_{\text{max}} \cdot X^h/(K_d^h + X^h)
\]

where B_{max} is the maximum specific binding; K_d is the concentration of apoA-I fibrils needed to achieve a half-maximum binding at equilibrium expressed, and h is the Hill slope.

Measurement of Mitochondrial Membrane Potential—Mitochondrial membrane potential was analyzed by using a mitochondrial membrane potentially sensitive fluorophore, tetramethylrhodamine, ethyl ester (TMRE) (MitoPT assay kit, ImmunoChemistry Technologies, Bloomington, MN). Briefly, CHO and pgsD-677 cells were plated on a poly-L-lysine-coated glass, cultured for 12 h, and treated with apoA-I amyloid (1 μM) for 12 h. After washing with PBS twice, the cells were loaded with TMRE (10 nM) at 37 °C for 20 min in the dark. The TMRE fluorescence was acquired by excitation wavelengths of 555 nm using an LSM710 confocal microscopy (Carl Zeiss MicroImaging GmbH, Jena, Germany). Mean fluorescence intensity was determined by using the ImageJ software (National Institutes of Health, Bethesda).
Assessment of Lysosomal pH—Lysosomal pH was assessed by using LysoSensor Yellow/Blue DND-160 (Thermo Fisher Scientific). Briefly, CHO and pgsD-677 cells were treated with apoA-I amyloid (1 μM) for 12 h followed by LysoSensor (1 μM, 3 min). The specimens were examined under an LSM 710 confocal laser microscope following the manufacturer's instructions. The red signal represents an acidic condition. For quantification, images were analyzed by using the ImageJ software.

Dot Blot for Detection of Highly Sulfated HS Domains—CHO cells were cultured and treated with 100 mM sodium chlorate as described above. Whole cell lysates were prepared, and aliquots (1 μl) were spotted on a nitrocellulose membrane. After being dried, the membrane was incubated with the RB4CD12 antibody, a phage display-derived anti-heparan sulfate antibody, followed by a horseradish peroxidase-conjugated monoclonal anti-VSV antibody (Sigma). The bound antibodies were visualized with ImmunoStar LD and examined by using a LAS-3000 mini-luminescent image analyzer.

Statistical Analysis—Data were analyzed via one-way analysis of variance, including the appropriate variables, followed by Dunnett's test or unpaired Student's t test. Results were regarded as significant when p < 0.05.

Results

Cellular Interaction of ApoA-I<sub>lowa</sub> Amyloid Requires HS—To assess the role of cellular HS in the interaction between apoA-I<sub>lowa</sub> amyloid and mammalian cells, we used CHO cells and their variant pgsD-677 cells, which have defective HS biosynthesis (40). CHO cells showed efficient interaction with apoA-I<sub>lowa</sub> amyloid that had been added to conditioned medium (Fig. 1A). Quantitative dot blot also showed that cellular interaction of both apoA-I<sub>lowa</sub> amyloid and WT apoA-I amyloid in pgsD-677 cells was reduced to 30–50% of the wild-type CHO cells (Fig. 1A). In the immunofluorescence study, CHO cells demonstrated substantial apoA-I amyloid staining signals, whereas pgsD-677 cells had subtle signals (Fig. 2). As an interesting finding, apoA-I<sub>lowa</sub> amyloid staining occurred close to cell membranes. The apoA-I signals did not co-localize with EEA1, a marker for endocytosis in which HSPGs reportedly act as a receptor for infectious prions (41). Cellular interaction of apoA-I<sub>lowa</sub> amyloid with CHO cells showed a dose-dependent manner (Fig. 1B).

To assess how much apoA-I<sub>lowa</sub> amyloid associates with the cell surface, we degraded the cell surface amyloid by trypsinizing the cells before preparing a whole cell lysate. As shown in Fig. 1B, apoA-I reactivity had almost completely disappeared by trypsinization. To further investigate whether apoA-I<sub>lowa</sub> amyloid was internalized or not, we looked at degradation pathways of apoA-I<sub>lowa</sub> amyloid. When cells were treated with apoA-I<sub>lowa</sub> amyloid for 12 h, washed with PBS, and further cultured in a fresh medium for 12 h, apoA-I signals were greatly diminished (Fig. 1D), which suggests that apoA-I<sub>lowa</sub> amyloid was degraded. However, the reduction in apoA-I signals was attenuated by culturing the cells in the presence of chloroquine (Fig. 1D), which is a weak base and impairs the function of lysosomes by neutralizing them (42). These results indicate that some portions of apoA-I<sub>lowa</sub> amyloid were internalized and degraded via a lysosomal pathway.

ApoA-I<sub>lowa</sub> Amyloid Cytotoxicity Depends on Cellular HS—We next studied the cytotoxicity of apoA-I<sub>lowa</sub> amyloid by means of the well established MTT assay, which estimates cell numbers on the basis of metabolic activity (37). Cells treated with 1 μM apoA-I<sub>lowa</sub> or WT apoA-I amyloid had reduced cell viability to 70% of the control level. These viability reductions were attenuated in the HS-deficient pgsD-677 cells treated with these amyloids (Fig. 1E). Thus, cytotoxicity of apoA-I<sub>lowa</sub> amyloid depended on cellular HS.

ApoA-I Amyloid Induces Mitochondrial Depolarization and Lysosomal Dysfunction—As MTT reduction occurs at least partly in the mitochondria (43), we asked whether apoA-I amyloid induces mitochondrial depolarization in cultured cells by using a mitochondrial potentially sensitive fluorescent dye, TMRE (44). As shown in Fig. 2, A and B, both apoA-I<sub>lowa</sub> and WT apoA-I amyloid decreased mitochondrial membrane potentials in CHO cells to 10–20% of the control level, indicating that apoA-I amyloid triggers mitochondrial dysfunction.

The reduction in mitochondrial membrane potentials in amyloid-treated pgsD-677 cells was 70–80% of the control level (Fig. 3, A and B). As lysosomal dysfunction has been linked to the loss of mitochondrial inner membrane potential (45), we next examined lysosomal functions in response to apoA-I amyloid. The number of acidic compartments such as lysosomes was decreased to 40–50% of the control level by treating cells with apoA-I amyloid (Fig. 4, A and B). These results indicate that apoA-I amyloid exerts its cytotoxicity by inducing mitochondrial depolarization and lysosomal dysfunction. Consistent with the results in the MTT assay (Fig. 1E) and the mitochondrial depolarization assay (Fig. 3), dysfunction in the lysosomes was markedly mitigated in pgsD-677 cells (Fig. 4B).

Exogenously Added Sulfated GAGs Attenuate ApoA-I<sub>lowa</sub> Amyloid Cellular Interaction and Cytotoxicity—Heparin, a structural analogue of HS, consists mostly of highly sulfated domains (46). We investigated whether heparin could inhibit cellular interaction and cytotoxicity of apoA-I<sub>lowa</sub> amyloid. Cells were treated with apoA-I<sub>lowa</sub> amyloid at 1 μM to detect cellular interaction in the absence or presence of an excess amount of heparin (5 μg/ml in conditioned medium). The whole cell lysate prepared from amyloid-treated CHO cells showed multiple anti-ApoA-I-positive bands within the range of 20 to >200 kDa in Western blotting and a dot in dot blotting (Fig. 5A). The lysates of CHO cells that were treated with amyloid plus heparin did not manifest this dot (Fig. 5A), which indicated that heparin suppressed the cellular interaction of apoA-I<sub>lowa</sub> amyloid. The immunofluorescence study supported these findings (Fig. 6).

We also used a nitrocellulose filter binding assay to determine whether apoA-I<sub>lowa</sub> amyloid could interact with heparin (39). Fig. 5B shows the FITC signals that were seen when a mixture of FITC-labeled heparin and apoA-I<sub>lowa</sub> amyloid, not apoA-I<sub>lowa</sub> amyloid or FITC-labeled heparin alone, was blotted on the filter. We confirmed that apoA-I<sub>lowa</sub> amyloid was trapped on the filter by subsequently probing apoA-I<sub>lowa</sub> amyloid with an anti-ApoA-I antibody (Fig. 5B, lower panel). To calculate the affinity of apoA-I<sub>lowa</sub> amyloid to heparin, we performed an enzyme linked immunosorbent assay (ELISA) by immobilizing heparin-BSA conjugates. The dissociation
constant \( (K_d) \) of apoA-I\textsubscript{Iowa} amyloid for heparin was \( 2.3 \mu M \) (Fig. 5C).

We also investigated the effects of other polyanions, HS and hyaluronic acid (HA), on apoA-I\textsubscript{Iowa} amyloid cellular interaction and cytotoxicity. As expected, HS (5 \( \mu g/ml \)) interfered with cellular interaction of apoA-I\textsubscript{Iowa} amyloid (Fig. 5D). HA is the only GAG member that is not sulfated (47). We next tested whether HA could interfere with cellular interaction of apoA-I\textsubscript{Iowa} amyloid. As shown in Fig. 5E, HA (5 \( \mu g/ml \)) had no effect on cellular interaction of apoA-I\textsubscript{Iowa} amyloid, which implies that sulfate groups of HS might be important for cellular interaction of apoA-I\textsubscript{Iowa} amyloid. Accordingly, heparin and HS, but not HA, reversed a decrease in cell viability induced by apoA-I\textsubscript{Iowa} amyloid (Fig. 5F).

Elimination of Cell Surface HS or Inhibition of HS Sulfation Reduces Interaction of ApoA-I\textsubscript{Iowa} Amyloid with CHO Cells—Xylose and its derivatives stimulate synthesis of xylose-linked GAGs in mammalian cells (48). These xylose-primed GAG chains are secreted into culture media. To see whether HS release from the cell surface could reduce cellular interaction of the amyloid, we treated cells with \( \beta \)-xyloside (2.5 mM) for 24 h before incubation with apoA-I\textsubscript{Iowa} amyloid. As Fig. 7 illustrates, \( \beta \)-xyloside reduced the number of apoA-I\textsubscript{Iowa} amyloid-positive cells to 40% of the nontreated level.

Because GAGs, including HS, are modified with sulfate groups and HA did not interfere with cellular interaction of apoA-I\textsubscript{Iowa} amyloid, we next investigated whether interaction of apoA-I\textsubscript{Iowa} amyloid with CHO cells required sulfate moieties of HS. Sodium chlorate is a metabolic inhibitor of the biosyn-
thesis of 3′-phosphoadenosine-5′-phosphosulfate, which is the sulfate donor for sulfation, and disrupts sulfation of HS (49). We confirmed the efficacy of sodium chlorate treatment by using a dot blot with RB4CD12, which is a phage display antibody that recognizes highly sulfated domains of HS (Fig. 8A) (50). When we treated cells with 100 mM sodium chlorate for 24 h and then incubated them with apoA-I\textsubscript{Iowa} amyloid, the number of apoA-I-positive cells was markedly reduced (Fig. 8, B and C).

**Sulf-1 and Sulf-2 Interfere with Interaction between ApoA-I\textsubscript{Iowa} Amyloid and Cells**—We recently reported that highly sulfated domains of HS accumulated in cerebral A\textsubscript{\beta} plaques in patients with AD or mouse models of AD and that these domains were degradable by human Sulf-1 and Sulf-2 (26). We also found that CHO cells constitutively produced and expressed HS containing highly sulfated domains and that the H\textsubscript{Sulf}s substantially reduced these domains to 25–50% of the mock-transfected cell level by removing C-6 sulfate groups when stably or transiently expressed (32). Therefore, we next investigated the possible role of highly sulfated domains in cellular interaction of apoA-I\textsubscript{Iowa} amyloid by taking an advantage of CHO cells that stably express human Sulf-1 or human Sulf-2, CHO-H\textsubscript{Sulf}-1 and CHO-H\textsubscript{Sulf}-2, respectively. We precultured these cells in Opti-MEM reduced-serum medium for 24 h to

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**FIGURE 2. Cellular interaction of apoA-I amyloid is dependent on HS in CHO cells.** A and B, CHO and pgsD-677 cells were plated on poly-L-lysine-coated cover glasses and incubated with 1 \( \mu \text{M} \) apoA-I amyloid, after which cells were permeabilized and stained with an anti-apoA-I antibody (red) and an anti-EEA1 antibody (green). DAPI counterstaining is shown in blue. Images at low (A) and high (B) magnifications are shown. Scale bar, 50 \( \mu \text{m} \) (A) and 10 \( \mu \text{m} \) (B).
optimize the effects of the Sulfs on HS (32), and we evaluated the interaction of apoA-I_{Iowa} amyloid with these cells. Both CHO-HSulf-1 and CHO-HSulf-2 cells manifested reduced cellular interaction of apoA-I_{Iowa} amyloid (Fig. 9A). The numbers of apoA-I_{Iowa} amyloid-positive transfectant cells were significantly reduced to 50–60% of the wild-type CHO level (Fig. 9B).
These results suggest that highly sulfated domains of HS on the cell surface are critical for interaction of apoA-I\textsubscript{Iowa} amyloid with the cell surface. Thus, apoA-I\textsubscript{Iowa} amyloid cytotoxicity was attenuated in these transfectants (Fig. 9C).

**Discussion**

HS has been shown to be associated with various amyloid deposits (51), which suggests that HS plays a role in pathogenesis or pathological processes of amyloidosis. Indeed, HS has been implicated in interaction and toxicity of different types of amyloid. For example, cellular uptake of Aβ in AD reportedly depends on GAGs, including HS, and cellular HS may mediate internalization and toxicity of Aβ (29, 30).

In agreement with these studies, results of our immunoblot and immunocytochemistry assays showed that cellular interaction of apoA-I\textsubscript{Iowa} amyloid decreased markedly in pgsD-677 cells, which are HS-deficient CHO cells, compared with wild-type CHO cells. These findings demonstrate that interaction of apoA-I\textsubscript{Iowa}...
amyloid with cultured cells depends on cellular HS. The fact that the cytotoxicity of apoA-I\textsubscript{iowa} amyloid was attenuated in pgsD-677 cells indicates that a reduced cellular interaction of apoA-I\textsubscript{iowa} amyloid appears to correlate with attenuation of the cytotoxicity of apoA-I\textsubscript{iowa} amyloid. Cellular interaction and cytotoxicity of apoA-I\textsubscript{iowa} amyloid were also reduced by addition of an excess amount of heparin and HS but not HA. Heparin and HS likely decreased the cytotoxicity by competing with the interaction of apoA-I\textsubscript{iowa} amyloid with cellular HS. Some portions of HSPG can be shed from the cell surface, which generates soluble HSPG (52). Inasmuch as all the β-xlyoside-primed GAG chains are secreted into culture medium, HS in the proteoglycan form can be eliminated by treating cells with β-xlyoside (48). Indeed, we determined that treatment of CHO cells with β-xlyoside reduced the amount of cellular apoA-I\textsubscript{iowa} amyloid. This finding together with the observations that sulfated GAGs interfered with cellular interaction of apoA-I\textsubscript{iowa} amyloid strongly suggest a critical role of cell surface HS in cellular interaction of apoA-I\textsubscript{iowa} amyloid. Furthermore, we found that cellular interaction of apoA-I\textsubscript{iowa} amyloid decreased

**FIGURE 6.** Heparin attenuates cellular interaction of apoA-I\textsubscript{iowa} amyloid. A and B, CHO cells were plated on poly-L-lysine-coated cover glasses and incubated with 1 \( \mu \)M apoA-I\textsubscript{iowa} amyloid with or without heparin (5 \( \mu \)g/ml), after which cells were permeabilized and stained with antibodies, and bound antibodies were visualized. DAPI counterstaining is shown in blue. Images at low (A) and high (B) magnifications are shown. Scale bar, 50 \( \mu \)m (A) and 10 \( \mu \)m (B).

**FIGURE 7.** β-Xyloside inhibits cellular interaction of apoA-I\textsubscript{iowa} amyloid. A, CHO cells were plated on poly-L-lysine-coated cover glasses, treated with 2.5 mM β-xlyoside, incubated with 1 \( \mu \)M apoA-I\textsubscript{iowa} amyloid, permeabilized, and stained with anti-apoA-I antibody, after which bound antibodies were visualized. DAPI counterstaining is shown in blue. Scale bar, 50 \( \mu \)m. B, graph shows quantification of amyloid-positive cell numbers. Data are means ± S.E. of three independent experiments. ***, \( p < 0.0001 \) versus β-xlyoside (–).**
after treatment of cells with sodium chlorate, which inhibits all sulfation modifications in cells (49). Thus, interactions of apoA-I_{lowa} amyloid with cells depend on sulfated HS on the cell surface. Although trypsinization eliminated the apoA-I signals almost completely, the observations that apoA-I fibrils were degraded via a lysosomal pathway and induced lysosomal dysfunction strongly suggest that some portions of apoA-I fibrils were internalized. A recent study has reported that lysosomal dysfunction underlies the cardioxicity in amyloid cardiomyopathy (53). Our results suggest that the lysosomal pathway also may play a critical role in pathogenesis of AApoAI amyloidosis. The reason why we could not detect intracellular EEA1-apoA-I signals is unclear. Figuring out the mechanism of apoA-I internalization and degradation is a forthcoming challenge.

Sulf-1 and Sulf-2 are extracellular endosulfatases that remove 6-O-sulfates on glucosamine residues in trisulfated disaccharides, which are components of highly sulfated domains of HS and heparin (17, 32). A knock-out study suggested that Sulf-1 and Sulf-2 contributed to the manifestation and maintenance of sulfation patterns in different organs, including the kidney (54), which is the major site of amyloid accumulation in apoA-I_{lowa} amyloidosis. Sulf-1 and Sulf-2 reportedly regulated several signaling pathways that were governed by molecular interactions between HS and protein ligands in extracellular spaces (16). We previously showed that highly sulfated domains of HS accumulated in amyloid plaques in patients with AD (26). Presumably, these highly sulfated domains could contribute to the pathology of amyloidosis. In this study, we demonstrated that inhibition of sulfation reduced cellular apoA-I_{lowa} amyloid and that CHO cells that stably expressed Sulf-1 or Sulf-2 showed less interaction with apoA-I_{lowa} amyloid compared with nontransfectant CHO cells. These results indicate that interactions of apoA-I_{lowa} amyloid with cells depend on HS sulfate moieties at the cell surface and that highly sulfated domains of HS play a role in the pathology of apoA-I_{lowa} amyloidosis. Our results also suggested that the activity level of endogenous Sulfs may be involved in the pathogenesis and/or progression of amyloidosis.

HS reportedly facilitated formation of various amyloid fibrils, including amylin (55), serum amyloid A (56), α-synuclein (57), prion protein (58), muscle acylphosphatase (59), Tau protein (60), and Aβ (61–63). In the case of apoA-I, we found that apoA-I_{lowa} amyloid interacted with heparin. Although the effects of heparin and HS on amyloid formation of the monomer apoA-I_{lowa} was subtle,3 our results clearly demonstrated that HS mediated cellular interaction of apoA-I_{lowa} amyloid. In one study, a patient with apoA-I_{lowa} amyloidosis manifested peripheral neuropathy, peptic ulcer disease, and nephropathy and died of renal failure (64). Such renal failures are due to renal amyloidosis (64, 65). HSPGs are abundant in the glomerular basement membrane, contribute to glomerular function in multiple ways, and are important for charge-selective permeability of the glomerular filter (66, 67). Whether HS and its highly sulfated domains accumulate together with apoA-I amyloid in

3 S. Mikawa and H. Saito, unpublished data.
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vivo is yet unknown. However, given our finding that HS and its highly sulfated domains mediated the cytotoxicity of apoA-I_{lowa} amyloid in a culture system, we suggest that HS may play a critical role in inducing dysfunction in specific organs that are sites of deposition of apoA-I amyloid.

In summary, we showed that interaction of apoA-I_{lowa} amyloid and CHO cells depended on sulfate moieties within HS on the cell surface. Cell surface HS also mediated the cytotoxicity of apoA-I_{lowa} amyloid. Additional investigations to clarify the role of HS in apoA-I amyloidosis in vivo are needed. Our results with CHO cells stably expressing Sulf-1 or Sulf-2 are particularly important, because enzymatic remodeling of cell surface HS may be a useful approach for modulating the pathology of apoA-I amyloidosis.

Author Contributions—K. K. performed most of the experiments. K. N. designed the research, interpreted the data, wrote the paper, and takes full responsibility for the manuscript. K. U. interpreted the data, contributed experimental materials, supervised the entire project, and edited the manuscript. S. C. H. contributed experimental materials. M. M. and H. N. performed the degradation assay. S. M. contributed to preparation of apoA-I fragments. N. K. established the anti-apoA-I antibody. H. S. contributed reagents/materials/analysis tools and supervised the entire project. N. S. contributed reagents/materials/analysis tools.

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