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Annexin A2 Enhances Complement Activation by Inhibiting Factor H

Brandon Renner,* Hua Hua Tong,† Jennifer Laskowski,* Karen Jonscher,* Lindsey Goetz,* Rachel Woolaver,* Jonathan Hannan,* Yong Xing Li,† Dennis Hourcade,§ Matthew C. Pickering,§ V. Michael Holers,* and Joshua M. Thurman*

Factor H is a circulating protein that regulates activation of the alternative pathway (AP) of complement. Mutations and genetic variations of factor H are associated with several AP-mediated diseases, highlighting the critical role of factor H in AP regulation. AP-mediated inflammation is typically triggered by tissue injury or inflammation, however, and tissue injury can trigger AP activation in individuals with fully functional factor H. This suggests that factor H function is affected by local conditions within tissues. We hypothesized that inducible proteins impair the ability of factor H to locally control the AP, thereby increasing AP activation. We used purified murine factor H to immunoprecipitate binding partners from mouse kidneys. Using immunoaffinity liquid chromatography–mass spectrometry, we identified annexin A2 as a factor H binding partner. Further experiments showed that annexin A2 reduces the binding of factor H to cell surfaces. Recombinant annexin A2 impaired complement regulation by factor H and increased complement activation on renal cell surfaces in vitro and in vivo. In a murine model of acute pneumococcal otitis media, the administration of annexin A2 increased AP-mediated bacterial opsonization and clearance. In conclusion, the local production of annexin A2 within tissues suppresses regulation of the AP by factor H. Annexin A2 can contribute to AP-mediated tissue inflammation by locally impairing factor H function, but it can also improve complement-mediated bacterial clearance. The Journal of Immunology, 2016, 196: 000–000.

A fundamental task of the immune system is to discriminate between host tissue and invasive pathogens. The alternative pathway (AP) is spontaneously activated in the fluid phase and on cell surfaces. Regulatory proteins control AP activation on host cells but not on pathogens, thereby enabling the AP to recognize invasive pathogens. Factor H is an AP regulatory protein that circulates in plasma, but it can control AP activation on the surface of host cells through its interaction with anionic molecules and complement C3 fragments displayed on the cell membrane (1). In the absence of factor H (e.g., in factor H–deficient mice), spontaneous complement activation is observed in plasma and in the kidneys (2). The strong association of variations and mutations in the gene for factor H with the development of inflammatory disease demonstrates the importance of factor H in controlling AP activation in humans (3).

Although factor H is present in high concentrations throughout the body, factor H mutations are associated with diseases of specific organs, including the kidneys (4–7) and the eyes (8–11), suggesting that these tissues are particularly dependent upon local factor H function for controlling AP-mediated injury. Even in patients with congenital factor H mutations, AP-mediated injury is usually triggered by systemic illness or by tissue injury (e.g., during transplantation) (12). It is also noteworthy that the AP contributes to disease even in patients who do not have factor H mutations. For example, clinical and experimental evidence indicates that AP activation causes injury after renal ischemia (13, 14), focal segmental glomerulosclerosis (15, 16), ANCA-associated vasculitis (17, 18), and lupus nephritis (19). These observations suggest that factor H is crucial for preventing AP-mediated injury to the host but that factor H function is affected locally within tissues by illness and other stressors.

We hypothesized that inducible factors expressed within tissues can interfere with factor H function. To test this hypothesis, we performed immunoprecipitation experiments in which biotinylated factor H was used to capture factor H binding partners from kidney tissue lysates. Tandem mass spectrometry identified one of the coimmunoprecipitated proteins as annexin A2. Annexin A2 was shown previously to associate with factor H, regulate the alternative pathway (AP), and contribute to the development of several AP-mediated diseases. We hypothesized that annexin A2 modulates AP activation by binding factor H and impairing its ability to control AP-mediated injury.

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Abbreviations used in this article: aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway; CMS, carbosmyethyl dextran; GAG, glycosaminoglycan; I/R, ischemia/reperfusion; KC, keratinocyte chemoattractant; rA2, recombinant murine annexin A2; siRNA, small interfering RNA; TEC, tubular epithelial cell.

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Materials and Methods

Study approval

C57BL/6 mice were purchased from The Jackson Laboratory. Mice with targeted deletion of the gene for factor H were generated as previously described (2). Mice were housed and maintained in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. The renal ischemia/reperfusion (I/R) protocol was approved by the University of Colorado Anschutz Center for Laboratory Animal Care, and the otitis media model was approved by the Ohio State University Animal Care and Use Committee.

Animal protocols

Renal I/R. Male C57BL/6 mice (The Jackson Laboratory) were used for the in vivo experiments. Ten- to twelve-week-old mice weighing 20–25 g were anesthetized i.p. with 60 mg/kg ketamine plus 10 mg/kg xylazine (both from Vedco) and euthanized. The kidney was isolated, and the renal pedicles were located and isolated by blunt dissection as previously described (13). The pedicles were clamped with surgical clips (Milteix Instrument), and occlusion of blood flow was confirmed by visual inspection of the kidneys. The clamps were left in place for 24 min and then released. The kidneys were excised and placed in ice-cold protease-free saline and then flash-frozen from were cut with 4-0 silk (United States Surgical). Sham surgery was performed in an identical fashion, with the exception that the renomed pedicles were not clamped. The mice were volume resuscitated with 0.5 ml normal saline and kept in an incubator at 29°C for 2 h to maintain body temperature during recovery. After 8 or 24 h the mice were anesthetized, and blood was obtained by cardiac puncture. Laporatomy was performed, and the kidneys were excised and snap-frozen in liquid nitrogen and creatinine was measured using an Alfa Wasserman ACE Chemistry Analyzer.

Factor H–deficient mice. Mice with a targeted deletion of the gene for factor H were generated as previously described (2) and were back-crossed for nine generations onto a C57BL/6 background. Heterozygous mice were used for the in vivo experiments. Otitis media model. Mice were anesthetized by i.p. injection with ketamine hydrochloride (20 mg/kg body weight) and xylazine (5 mg/kg). Acute otitis media was induced by direct bilateral transtympanic inoculation with 1 × 10^9 CFU opaque or transparent Streptococcus pneumoniae in sterile pyrogen-free saline, as previously described (21, 22). Mice were anesthetized and sacrificed 24 or 48 h post inoculation. The middle ear spaces were lavaged to quantitatively determine titers of S. pneumoniae. The middle ear lavage samples were cultured overnight at 37°C on Columbia CNA agar plates in an incubator supplemented with humidity and 5% CO2. CFU/ml was determined by a standard dilution assay and plate counting.

Protein purification and production

Factor H. An affinity column for factor H was made by ligating polyclonal goat Ab for human factor H (Quidel) to CNBR Sepharose (Amersham Biosciences), according to the manufacturer’s instructions. Plasma was collected from C57BL/6 mice and passed over the column. After washing the column with PBS (pH 7.4), factor H was eluted with 5 M LiCl. The buffer was exchanged by Ultrafree-MC (Amersham) for 300,000 MW cutoff columns. Factor H was passed through a Hiload Superdex 200 size exclusion column (GE Amersham). The purity and mobile phase was returned to the initial conditions after 10 min.

Western blot and Far-Western blot analyses

For analysis of kidney proteins, kidneys were homogenized in RIPA lysis buffer containing 1% Triton X-100, 0.5% deoxycholic acid, 150 mM NaCl, 20 mM β-glycerophosphate, 20 mM Tris·HCl (pH 8), 5 mM EGTA, 3 mM MgCl2, 0.1% SDS, 1 mM DTT, 50 µM Na3VO4, and one tablet of complete, EDTA-free, protease inhibitor mixture (Roche Applied Science). Homogenates were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was collected. Protein concentrations for kidney samples were determined using the Bio-Rad protein assay. One hundred micrograms of protein from each kidney was resolved by electrophoresis with a 10% Bis-Tris polyacrylamide gel (Invitrogen) and transferred to a nitrocellulose membrane. Factor H was detected in tissue lysates or in solution using a polyclonal goat anti-human factor H Ab (Quidel) diluted 1:100. Annexin A2 was detected in kidney lysates or in solution using a polyclonal anti-human annexin A2 Ab (Proteintech) diluted 1:2000. Densitometry was performed using ImageJ software. The relative expression of annexin A2 in each sample was determined by dividing the band density by that for a-actin. Appropriate secondary Abs (Jackson ImmunoResearch) were used. To detect the binding of factor H to proteins in kidney lysates (Far-Western analysis), purified factor H was biotinylated with Sulfo-NHS-Biotin (Pierce), according to the manufacturer’s instructions. The sample proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with 1 µg/ml biotinylated factor H in PBS (pH 7.4). The binding of factor H to protein bands was detected with Streptavidin-HRP (Zymed) diluted 1:500 in PBS, followed by ECL.

Immunoprecipitation of factor H binding partners

Purified factor H was biotinylated using Sulfo-NHS-Biotin (Pierce). Three micrograms of factor H was added to 100 µg kidney protein lysate. Streptavidin Beads (0.5 mg Dynabeads M-280) were added to the mixture and incubated for 30 min at room temperature. A magnet was used to separate the beads, and the beads were washed four times with PBS. Precipitated proteins were separated on a Bis-Tris gel and stained with BioSafe Coomassie Stain (Bio-Rad).

The ~39-kDa gel band was excised and digested using trypsin (Promega Gold) following reduction with 1.5 mg/ml DTT and alkylation with 10 mg/ml iodoacetamide. Resultant peptides were extracted with acetonitrile and 1% formic acid.

Protein identification by nano-scale liquid chromatography/nanoelectrospray quadrupole ion trap tandem mass spectrometry

Tryptic peptides were analyzed by reverse-phase nano-scale liquid chromatography/nanoelectrospray quadrupole ion trap tandem mass spectrometry using an Agilent 1100 HPLC system equipped with a nanopump coupled to an Agilent LC/MSD Trap XCT Plus (Agilent Technologies) mass spectrometer. Peptides were loaded onto a Zorbax C18 column (75 µm inner diameter × 10 cm, 300 Å porosity, 5 µm particles) (Agilent Technologies) for 2 min using a MicroWell plate autosampler and a capillary pump delivering a flow of 5 µl/min with buffer A (0.1% formic acid). Peptides were eluted by a gradient solvent A (0.1% formic acid) and solvent B (90% acetonitrile, 0.1% formic acid) at a flow rate of 300 nℓ/min. The gradient was ramped from 3 to 8% solvent B in 1 min, from 8 to 45% solvent B in 85 min, and finally to 90% B in 5 min; the mobile phase was returned to the initial conditions after 10 min.

Spray was established using 8-µm inner diameter emitters (New Objective, Woburn, MA) and a typical capillary voltage of 1600 V. Spectra were collected over 350–1800 m/z. Three fragment masses were collected for the three most abundant m/z values. Subsequently, those m/z values were excluded from the analysis for 1 min and the next three most abundant m/z values were selected for fragmentation to enable analysis of lower abundance peptide ions.

The Spectrum Mill database search algorithm (Agilent Technologies) was used to search the UniProt database, using the taxonomy filter for mouse. Peptides were used for the search included the monoisotopic mass, a peptide mass tolerance of 1.2 Da, and a fragment ion mass tolerance of 0.6 Da. Furthermore, tryptic peptides were only allowed two missed cleavages, and carbamidomethylation of cysteine was selected as a fixed modification. Posttranslational methylation of cysteine was selected as a fixed modification. Posttranslational methylation of cysteine was selected as a fixed modification.
 modifications (glycosylations and/or phosphorylations) as possible variable peptide modifications were not included in the search parameters.

Database matches were validated by reverse database scoring using Spectrum Mill software. Proteins with Spectrum Mill scores > 10 and peptide scores > 10, as well as those that scored a percent intensity of 70%, were used as thresholds for initial hit validation. Each identified protein required confirmation with at least two unique peptides.

For the most interesting candidates, peptide determinations used for protein identifications were validated manually, marking observed and theoretically expected mass ions. Water losses, evident when peptide fragments containing a serine or threonine were measured, were used to assist in sequence assignment validation.

**Immunofluorescence microscopy and immunohistochemistry**

Kidneys were snap-frozen in OCT compound (Sakura Finetek). Four-micron sections were cut with a cryostat and stored at −70°C. The slides were fixed with acetone. C3 was detected with an FITC-conjugated anti-mouse C3 Ab (MP Biomedicals) diluted 1:150. A mAb that recognizes only the iC3b and C3d fragments of C3 was also used (23). Annexin A2 in kidney sections was detected by immunohistochemistry using an anti-annexin II Ab (clone Z014) and a SuperPicture Polymer Detection Kit (both from Invitrogen).

**Annexin A2–Factor H binding assays**

**Fluid-phase binding.** Purified murine factor H and recombinant annexin A2 were separated by size-exclusion chromatography using a Superdex 200 column (GE Amersham), and the eluate was divided into five fractions. The eluted fractions were concentrated, and factor H and annexin A2 were detected by Western blot analysis.

**Solid-phase binding.** To examine solid-phase binding of annexin A2 and factor H, 800 ng recombinant annexin A2 was adsorbed overnight to round-bottom plates (Immunol). The plates were blocked with 3% milk in TBS (pH 7.5) for 2 h. Factor H (100 μM) at 20 μg/ml in TBS with 2 mM Ca) was added to the wells, and the plates were incubated for 3 h on a rocker at room temperature and then washed three times with TBS. Bound factor H was detected by incubating the plates with 100 μl/well anti-giant factor H (Quidel) diluted 1:10 in 3% milk blocking buffer for 1 h with rocking. The plates were washed five times, and 100 μl 1:1000 anti-giant HRP in 3% milk was added to each well. After incubating the plates for 1 h, they were washed and developed with One-Step Ultra TMB-ELISA Substrate (Pierce). The reaction was stopped after ~20 min with 100 μl M sulfuric acid, and absorbance was read at 450 nm.

To study the effect of annexin A2 on the surface of sheep erythrocytes, Ab-sensitized erythrocytes were purchased from CompTech. C3bBbP was generated on the cell surface, and the effects of annexin A2 on decay of the convertase by factor H were studied as previously described (23). The interaction between annexin A2 and factor H was also examined using a Biacore 3000 (Biacore) at the University of Colorado Biophysics Core. Recombinant factor H was immobilized on a carboxymethyl dex-

Trans (CM5) chip using random amine coupling with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/N-hydroxysulfosuccinimide as the activating reagent and at a concentration of 50 μg/ml in 100 mM sodium acetate (pH 5). Approximately 7000 RU factor H was immobilized, and the remaining activated groups on the surface of the chip were blocked with 1-M ethanolamine solution (pH 8.5). Binding experiments were conducted in 10 mM HEPES, 150 mM NaCl, 0.005% P2O (pH 7.4), and the chip was regenerated between analyte injections with two 10-μl injections of 10 mM NaOH. Recombinant annexin A2 was injected at concentrations of 3 μM, 1 μM, 333 nM, 111 nM, and 37 nM for 3 min at a flow rate of 25 μl/min, and the dissociation of the resulting annexin A2–factor H complexes was monitored for 10 min. All injection were performed in triplicate to verify reproducibility, and all data were double referenced using a blank flow cell and a blank injection of buffer to account for nsspecific binding and baseline drift, respectively. Data were fit using a 1:1 Langmuir binding model, and data analysis was performed using SCRUBBER-2 software (distributed by Dr. David Myszka, University of Utah Center for Biomolecular Interactions, Salt Lake City, UT).

**Suppression of annexin A2 expression with small interfering RNA**

To suppress annexin A2 expression, cells were treated with three small interfering (siRNA) plasmids or with a control plasmid (F1514943, F1514944, F1514945), according to the manufacturer’s instructions (OriGene). Immortalized murine tubular epithelial cells (TECs) were grown in culture until ~80% confluent and were then transfected with the siRNA using Lipofectamine 2000 and Opti-MEM medium (Life Technologies) under selection with puromycin for 7 d. Suppression of annexin A2 expression was confirmed by Western blot analysis and by flow cytometry, and clones with reduced annexin A2 expression were chosen for further analysis.

**Quantitative RT-PCR**

RNA was isolated from tissue using TRIzol reagent (Life Technologies), according to the manufacturer’s instructions. cDNA was generated using 1 μg RNA using a High Capacity cDNA Reverse Transcriptase kit from Applied Biosystems. The cDNA abundance for annexin A2 and actin (as a loading control) was measured using Power SYBR Green Master Mix (Applied Biosystems) and a Roche light cycler machine. The primers used to detect annexin A2 were 5'-CTCTCCTACGGGTCAGC3'- (forward) and 5'-GCTGTTCATGATTCAGAGCA-3' (reverse), whereas those used to detect actin were 5'-GCTGTATCTCCATCCTACG-3' (forward) and 5'-CCAGTTGGAACAATGCCATG-3' (reverse).

**Flow cytometry**

Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). Cells analyzed included murine TECs, murine microvascular endothelial cells (MS1 cells; American Type Culture Collection), murine mesangial cells (SV40 MES 13; American Type Culture Collection), and Jurkat cells. C3 deposition on cells was determined using a FITC-labeled goat anti-mouse C3 Ab (MP Biomedicals; 12 μg/ml). Annexin A2 expression on cell surfaces was detected using Invitrogen clone Z014 diluted 1:50, followed by Cy-5-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) diluted 1:200. For some binding experiments, recombinant factor H or recombinant annexin A2 was fluorescently labeled with DyLight 650 (Thermo Fisher Scientific). In some experiments, the populations of necrotic cells were identified by staining with propidium iodide.

**ELISAs**

C3a levels were measured by ELISA using mAbs and standards from BD Biosciences, according to the manufacturer’s instructions. IL-6 and keratinocyte chemotactic factor (KC) were measured using kits from R&D Systems, according to the manufacturer’s instructions.

**FIGURE 1.** Factor H does not prevent complement activation in the kidney after IR. (A) Kidney sections were immunostained for C3b and iC3b/C3d. The lower panels display enlarged views of C3 fragment deposition at the corticomedullary junction. The regions shown are indicated with boxes in the top panels. In some locations C3b is incompletely converted to C3d (arrows) (original magnification ×100). (B) Lysates of the outer medullae of kidneys were probed for factor H by Western blot analysis. Factor H (indicated with a dashed line) was detected in the kidneys of unmanipulated C57BL/6 mice and in kidneys subjected to ischemia and varying times of reperfusion.
considered statistically significant. Results are reported as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (A) R N A

Statistics  

Multiple-group comparisons were performed using ANOVA with posttest using the Tukey multiple-comparison test. Comparisons between two groups were performed with the Student t test. A p value < 0.05 was considered statistically significant. Results are reported as mean ± SEM.

Results  

Cofactor activity is lost in the injured kidney  

The AP is activated in the renal tubulointerstitium after I/R (13). During complement activation, the protein C3 is converted to C3b. C3b is subsequently cleaved (inactivated) by factor I, generating iC3b. To perform this cleavage, factor I requires a cofactor protein. In the mouse, the proteins Crry (25) and factor H (26) can serve as cofactors. We stained tissues from sham-treated and postischemic kidneys with an Ab that recognizes C3b and a mAb that only recognizes iC3b and/or C3d (23) (Fig. 1A). C3b is deposited in renal tubules after I/R, reflecting complement activation in the tubulointerstitium. However, C3b is not completely converted to iC3b/C3d, indicating limited cofactor activity in the renal tubulointerstitium.

We showed previously that expression of Crry decreases on TECs after I/R and that complement activation begins within 6 h of reperfusion (27). Because factor H can also serve as a cofactor, we

performed Western blot analysis for factor H in lysates made from the outer medullae of kidneys subjected to I/R. Factor H was present in unmanipulated ischemic kidneys, and levels persisted during reperfusion (Fig. 1B). Thus, complement activation occurs in the renal tubulointerstitium, even in the presence of factor H. Factor H localizes to anionic surfaces enriched in anionic markers, such as glycosaminoglycans (GAGs), sialic acid glycans, and C3b (1, 28). Factor H was also detected within the kidneys of factor B–knockout mice subjected to renal I/R (data not shown), even though C3b is not deposited on the tubules after I/R in this mouse strain (13). This indicates that binding partners for factor H other than C3b are present within the postischemic kidney.

Factor H binds to the protein annexin A2 in the postischemic kidney

To identify potential binding ligands for factor H in the postischemic kidney, we performed Far-Western blots in which kidney lysates were probed with biotinylated factor H. Factor H bound to several proteins within the lysates, and binding to some bands was stronger in lysates from postischemic kidneys (Fig. 2A). Biotinylated factor H was also incubated with kidney lysates, and factor H was then isolated using streptavidin beads to coimmunoprecipitate binding partners. The isolated proteins were examined by gel electrophoresis, and multiple protein bands were visualized (Fig. 2B). Several bands appeared stronger in lysates made from postischemic kidneys, including a band at ∼39 kDa that was also seen in the Far-Western blot.

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FIGURE 4. Annexin A2 binds factor H and increases complement activation in the fluid phase. (A) Factor H was purified from mouse plasma by heparin chromatography, and annexin A2 was detected in samples generated from wild-type serum and in a commercial preparation of factor H; however, it was not seen in a sample generated from factor H–deficient (fH--) serum. Bands from two blots are shown. (B) Factor H purified from plasma and recombinant annexin A2 were passed over a size-exclusion column. Annexin A2 was detected in all of the fractions of factor H purified from serum. When recombinant annexin A2 (rA2) was passed over the column in the absence of factor H, it did not elute in the higher molecular mass fractions (fractions 1 and 2; indicated with arrows). (C) Purified annexin A2 was added to normal mouse serum and incubated at 37°C for 30 min, and C3a in the supernatant was indicated with arrows. (D) Western blot analysis of plasma from mice injected with recombinant annexin A2 revealed greater cleavage of plasma C3 compared with mice injected with PBS as a vehicle control.

The ∼39-kDa band from the immunoprecipitation reaction was excised from the gel and digested using trypsin. Resultant peptides were analyzed by liquid chromatography–tandem mass spectrometry, and spectra were identified by database searching. This band yielded eight peptides, six of which were unique, that mapped to annexin A2; the protein search score was 75.13, and these peptides accounted for 17% of the protein amino acid sequence. Identification of three of the peptides was confirmed manually using their tandem mass spectra. The tandem mass spectrum for one of the peptides is shown (Fig. 2C). The peptide score was 18.91, and the percent intensity (a measure of how much of the ion current is accounted for by theoretically expected peaks) for this spectrum was 92.6%, suggesting that the dominant ions conform to those theoretically expected for this sequence (29). Thirteen other proteins were identified from this analysis, but all were either intracellular proteins or likely contaminants (e.g., keratin).

Factor H was shown to bind annexin A2 on the surface of apoptotic cells (20), and the expression of annexin A2 by TECs was shown to increase after renal I/R (30). We performed quantitative RT-PCR and found that mRNA levels for annexin A2 increased after I/R in our model (Fig. 3A), and Western blot analysis confirmed that levels of annexin A2 protein in the kidney increased after I/R (Fig. 3B). Immunoprecipitation experiments in which biotinylated factor H was used to pull-down annexin A2 demonstrated that factor H bound to more annexin A2 in lysates from posts ischemic kidneys than from sham-treated controls (Fig. 3C). Annexin A2 was detected in the outer medulla of postischemic mice by immunohistochemistry (Fig. 3D), the location where complement activation occurs after I/R (Fig. 1A).

Annexin A2 binds to factor H in the fluid phase and on surfaces

We purified mouse factor H from normal mouse serum using heparin chromatography, followed by size-exclusion chromatography. We then performed Western blot analysis of the purified factor H with an Ab for annexin A2 and found that annexin A2 was present in the sample. However, when factor H–deficient serum was subjected to the same heparin chromatography and size-exclusion chromatography protocols, annexin A2 was not purified (Fig. 4A). This demonstrates that annexin A2 and factor H in serum bind, and isolation of factor H from normal mouse serum also captures annexin A2. Annexin A2 was also detected in a commercially available sample of human factor H (Fig. 4A).

Factor H from normal mouse serum was purified using heparin chromatography and subsequently applied to a size-exclusion column. Eluted proteins were collected, pooled into five separate fractions, and analyzed by Western blot analysis (Fig. 4B). Factor H was detected in all of the pooled fractions after gel filtration (molecular mass range ∼25–2000 kDa). Annexin A2 was copurified from serum during the heparin chromatography, and it too was detected in high molecular mass fractions collected when the purified factor H was separated on the size-exclusion column. Recombinant annexin A2 (in which no factor H is present) was passed through the size-exclusion gel under the same conditions. This pure annexin A2 sample was only detected in fractions 3–5, corresponding to a molecular mass range ∼25–120 kDa.

To determine whether annexin A2 has a detectable functional effect on complement activation, 3.5 μg of recombinant annexin A2 was added to normal mouse serum (200 μl of 10% serum in PBS), representing an ∼3-fold molar excess of annexin A2 to factor H. The mixture was incubated at 37°C, and C3a was measured as a marker of complement activation in the serum. The addition of annexin A2 increased C3a production in the serum, indicating that annexin A2 promotes complement activation (Fig. 4C). In addition, 75 μg of recombinant annexin A2 was injected i.v. into wild-type mice, and plasma was collected after 24 h. Western blot analysis of the plasma for C3 demonstrated conversion of C3 to C3b (Fig. 4D).

To determine whether factor H binds to annexin A2 on solid surfaces, we immobilized annexin A2 on an ELISA plate.
Recombinant factor H bound to annexin A2 in this assay (Fig. 5A).

We also examined the interaction of the proteins using surface plasmon resonance. Factor H was immobilized on a CM5 chip, and various concentrations of annexin A2 were injected. Annexin A2 bound to the immobilized factor H with a dissociation constant $\sim 7\ \text{mM}$ (Fig. 5B).

Expression of annexin A2 by renal cells increases complement activation on the cell surface

C3b is deposited on the surface of murine renal TECs grown in culture after exposure to normal mouse serum, but we previously found that antagonism of factor H causes a slight increase in complement activation on the cell surface (31). We treated TECs with siRNA to suppress annexin A2 expression. The level of annexin A2 on the cell surface and in the cell supernatant decreased after treatment with the siRNA (Fig. 6A). Decreased expression of annexin A2 was associated with increased binding of factor H to the cell surface (Fig. 6B), indicating that expression of annexin A2 by the cells is associated with reduced binding of factor H to the cell surface. We then exposed the cells to normal mouse serum and measured C3b deposition on the cell surface by flow cytometry (Fig. 6C). Suppression of annexin A2 expression with siRNA decreased the amount of C3 deposited on the cell surface, demonstrating that annexin A2 produced by TECs increases complement activation on the cells.

In another set of experiments, cells were incubated with normal mouse serum in the presence or absence of additional recombinant murine annexin A2 (rA2). When murine TECs were incubated with recombinant annexin A2, C3b deposition on cells exposed to serum increased (Fig. 7A). However, if we replaced the supernatant prior to adding serum, no change in C3b deposition was seen, suggesting that unbound annexin A2 in the supernatant mediated

**FIGURE 6.** Silencing of annexin A2 reduces complement activation on TECs. The expression of annexin A2 by TECs was knocked down by treating the cells with siRNA for annexin A2 or with control siRNA. (A) Expression of annexin A2 was examined by flow cytometry. Treatment of the cells with siRNA for annexin A2 significantly reduced surface annexin A2. Annexin A2 in the supernatant was examined by Western blot analysis (inset). Annexin A2 was detected in the supernatant of cells treated with control siRNA, but levels were lower in the supernatant of cells treated with siRNA for annexin A2. (B) Clones with low annexin A2 expression were exposed to normal mouse serum, and the level of factor H bound to the cells was examined by flow cytometry. The amount of factor H on the cell surface was significantly higher when annexin A2 expression was reduced by treatment with siRNA. (C) Clones with low annexin A2 expression were exposed to normal mouse serum, and C3b deposition on the cells was measured by flow cytometry as a marker of complement activation. C3b deposition on the cell surface was lower on cells with reduced expression of annexin A2 than on cells treated with control siRNA. $^*p < 0.05, **p < 0.01.$

**FIGURE 7.** Annexin A2 increases complement activation on the surface of TECs and endothelial cells. (A) Murine TECs were incubated with normal mouse serum, and recombinant annexin A2 (inset) was added to the reaction. C3b deposition on the cells was measured by flow cytometry. The addition of annexin A2 increased C3b deposition on the cells ($n = 6$). (B) Viable and necrotic cell populations were identified using propidium iodide, and C3b deposition on these populations was examined by flow cytometry. More C3b was detected on the surface of necrotic cells than on viable cells. The addition of annexin A2 increased C3b deposition on both the viable and the necrotic cell populations. (C) We also tested whether annexin A2 affects complement activation on endothelial cells. There was a trend toward greater C3b deposition when the cells were exposed to serum in the presence of annexin A2 ($n = 3$). $^*p < 0.05, **p < 0.01, ***p < 0.001.$
the increase in C3b deposition. Increased C3b was seen on both viable and necrotic TECs, but C3b deposition was greatest on necrotic TECs treated with annexin A2 (Fig. 7B). The addition of rA2 to the reaction also increased C3b deposition on murine microvascular endothelial cells (Fig. 7C). Use of commercial purified bovine annexin A2 gave results with TECs that were similar to those obtained with the recombinant murine protein (results not shown).

Factor H and annexin A2 both bind to the surface of TECs (Fig. 8A, 8B). Yet, in the presence of annexin A2, less factor H bound to the cells. Thus, annexin A2 produced by the TECs (Fig. 6) or added directly to the cell supernatants (Fig. 8C) inhibits binding of factor H to the cells and increases AP activation on the cell surface. Although it is possible that some of the annexin A2 bound to the cell surface mediates binding of factor H to the cell membrane, the net affect of annexin A2 in the reactions is to reduce the amount of factor H bound to the cells.

The effect of annexin A2 on complement regulation appears to be cell specific. Recombinant annexin A2 did not increase complement activation on the surface of Jurkat cells (an immortalized human T cell line), but it did increase complement activation on the surface of ARPE cells (a human retinal epithelial cell line) when both cell types were exposed to human serum (data not shown).

Similarly, we performed an assay in which sheep erythrocytes are lysed by the AP when exposed to human serum. Purified factor H decreases cell lysis in this assay by accelerating the decay of the C3bBbP complexes on the sheep erythrocytes. However, we did not detect an effect of recombinant annexin A2 on factor H function in this assay (data not shown).

To further explore the mechanism by which annexin A2 promotes complement activation on the TEC surface, we incubated TECs with recombinant annexin A2 and serum from mice deficient in C4 (C4−/− mice; deficient in classical pathway activity). Annexin A2 increased C3 deposition on TECs exposed to serum from C4−/− mice (Fig. 9A), indicating that increased complement activation in the presence of annexin A2 does not require the classical pathway. However, treatment with a mAb to factor B that prevents AP activation (mAb 1379) (32) prevented the increase in C3 deposition in cells exposed to annexin A2 (Fig. 9B). Furthermore, the addition of 100 μg/ml of purified murine factor H to the reaction prevented the rA2-induced increase in C3 deposition (Fig. 9C). In contrast, when we added a peptide that prevents...
To determine whether annexin A2 promotes complement activation on nearby cell surfaces. This effect is likely mediated through its interaction with factor H because the addition of supraphysiologic concentrations of factor H reverses the effect, yet the addition of an antagonist of factor H (H19–20) has no further effect on complement activation.

Annexin A2 increases intrarenal complement activation

To determine whether annexin A2 promotes complement activation in vivo, we subjected mice to sham treatment or renal I/R. We then injected mice from each group with 75 μg of recombinant annexin A2 and sacrificed after 24 h. (A) Immunofluorescence microscopy demonstrated glomerular C3b deposition (original magnification ×400). Arrowheads indicate glomeruli. (B) Glomerular C3b deposits were significantly greater in mice injected with recombinant annexin A2 after renal I/R than in those injected with a vehicle control. *p < 0.05. Serum urea nitrogen (C) and serum creatinine (D) levels were not significantly different in mice injected with recombinant annexin A2 compared with vehicle controls.

Exogenous annexin A2 caused widespread C3b deposition in the kidneys of H+/− mice (Fig. 11). In addition to C3b deposition in the mesangium, C3b was deposited continuously along the renal tubules throughout the tubulointerstitium. The effects of annexin A2 were much more extensive in H+/− mice than in wild-type mice, demonstrating that these proteins have opposing roles in controlling complement activation in vivo.

Annexin A2 improves clearance of S. pneumoniae during acute otitis media

We showed previously that AP activation mediates opsonophagocytosis of the bacteria in this model (22). In vitro, more C3b was deposited on S. pneumoniae incubated with either normal human or murine serum when annexin A2 was added to the reaction (Fig. 12A, 12B), demonstrating that annexin A2 can also increase complement activation on the surface of bacteria. Annexin A2 did not increase C3b deposition on the bacteria when serum from factor B−deficient mice was used. Purified annexin A2 and factor H both bound to a subset of the bacteria (Fig. 12C, 12D). However, similar to what was seen with TECs, the addition of annexin A2 reduced binding of factor H to the surface of the bacteria.

To test whether annexin A2 affects bacterial clearance by the complement system in vivo, we injected annexin A2 (2.5 μg/ear) with S. pneumoniae into the middle ears of mice. Injection of annexin A2 improved clearance of the bacteria (Fig. 13A). Clearance of the bacteria was not improved in B−/− mice injected with annexin A2 (Fig. 13B), confirming that improved bacterial clearance in mice injected with annexin A2 is mediated by the AP. Although annexin A2 promotes complement activation, it decreased production of the inflammatory cytokines IL-6 (Fig. 13C) and KC (CXCL1; Fig. 13D) in mice with otitis media.

Discussion

Factor H is the most important regulator of the AP, and it is present at high concentrations in the plasma of healthy individuals (∼250 μg/ml) (20, 34). However, infections and aseptic tissue injury trigger AP activation, suggesting that AP regulation by factor H is...
When annexin A2 was added to NMS, the addition of annexin A2 did not reduce the bacteria was measured by flow cytometry and was significantly greater of factor H to the bacteria. Each experiment was performed three times. Recombinant annexin A2. The addition of annexin A2 reduced binding moniae type 6A was incubated with fluorescently labeled factor H and labeled annexin A2, which bound to a subset of the cells. (FIGURE 12. Annexin A2 increases clearance of S. pneumoniae in acute otitis media. (A) S. pneumoniae type 6A was incubated with 50% normal human serum (NHS), with or without the addition of annexin A2 (2 μg/ml). C3b deposition on the bacteria was measured by flow cytometry and was significantly greater when annexin A2 was added to the reaction. (B) S. pneumoniae type 6A was incubated with 50% normal mouse serum (NMS) or serum from factor B–deficient mice (fB/−/−). C3b deposition on the bacteria was measured by flow cytometry and was significantly greater when annexin A2 was added to NMS. The addition of annexin A2 did not increase C3b deposition on the bacteria when incubated with serum from fB−/− mice. (C) S. pneumoniae type 6A was incubated with fluorescently labeled annexin A2, which bound to a subset of the cells. (D) S. pneumoniae type 6A was incubated with fluorescently labeled factor H and recombinant annexin A2. The addition of annexin A2 reduced binding of factor H to the bacteria. Each experiment was performed three times. **p < 0.01, ***p < 0.001.) impaired or overwhelmed in injured tissues. In the current article, we report that production of the protein annexin A2 locally blocks regulation of the AP by factor H and increases complement activation.

The AP is activated in the kidneys of mice (13) and humans (14) after I/R, yet factor H is present in kidneys during the period of complement activation. Proteomic analysis revealed that the protein annexin A2 was present in the ischemic kidney and bound to factor H. Further experiments demonstrated that annexin A2 interfered with AP regulation by factor H and promoted AP activation on the surface of certain cell types. TECs treated with siRNA expressed reduced annexin A2 on the cell surface and in the supernatant. Decreased expression of annexin A2 was associated with increased binding of factor H to the cell surface and reduced C3b deposition on the cell. In contrast, the addition of recombinant annexin A2 to the cell supernatant decreased binding of factor H to the cell surface and increased C3b deposition on the cells. However, increased complement activation was not observed when the medium containing recombinant annexin A2 was replaced prior to addition of the serum. Therefore, although annexin A2 is expressed on cell surfaces and can bind to cell surfaces, its effect on AP activation is primarily mediated by binding to factor H in solution and preventing factor H from binding to cells. The effect of annexin A2 on complement activation proceeded via the AP, and supraphysiologic factor H was able to reverse the process.

The injection of mice with annexin A2 increased complement activation within the glomeruli. Complement activation was even more extensive when annexin A2 was injected into mice with partial deficiency of factor H (fH+/− mice). In these mice, C3b deposition was seen in the glomeruli and along the tubules throughout the kidney. However, we also found that injection of annexin A2 into the inner ears of mice improved the clearance of S. pneumoniae and reduced the production of proinflammatory cytokines in a model of acute pneumococcal otitis media. Opsonization of bacteria with C3 was greater in the presence of annexin A2. However, no effect was seen when annexin A2 was injected into fB−/− mice during acute pneumococcal otitis media, confirming that the antibacterial effect of annexin A2 requires an intact AP. Although annexin A2 may also trigger pathologic AP activation in the middle ear in other contexts, our in vitro and in vivo results suggest that increased eradication of bacteria facilitated by annexin A2 is associated with reduced production of inflammatory mediators during acute otitis media.

Annexin A2 is a Ca2+-regulated phospholipid-binding protein that has numerous intracellular and extracellular functions (35, 36). Extracellular annexin A2 functions as a surface-bound receptor for several molecules, including factor H (20), C1q (37), plasminogen (38), tissue plasminogen activator (39), and some pathogens (40, 41). Annexin A2 is also the target of autoantibodies in several diseases. Anti-phospholipid Abs recognize annexin A2 and block its fibrinolytic function (42), and anti-dsDNA Abs in patients with lupus react to annexin A2 (43). Interestingly, annexin A2 was identified within the lesions of several complement-mediated diseases, including age-related macular degeneration (43), nephrotic syndrome (44), and SLE (45).

FIGURE 13. Annexin A2 increases clearance of S. pneumoniae in acute otitis media. Survival of S. pneumoniae type 6A in the middle ear of WT mice (A) and fB−/− mice (B), with or without annexin A2, was examined. Each data point represents the mean S. pneumoniae (CFU/ml) in the middle ear lavage fluid samples. Concentrations of IL-6 (C) and KC (D) in the middle ear lavage samples at 24 and 48 h postinfection with S. pneumoniae type 6A, with or without annexin A2. Results are the mean concentrations of IL-6 and KC (± SEM) in the middle ear lavage samples from two duplicate wells from two separate experiments. *p < 0.05.
degeneration (44), dense deposit disease (45), and lupus nephritis (43). By binding C1q, annexin A2 may trigger classical pathway activation (37), and simultaneous inactivation of factor H would cause amplification through the AP. Whether endogenous annexin A2 plays a pathogenic role in tissue inflammation through its interaction with the complement system remains to be determined.

In recent years, significant progress has been made in our understanding of the role of the AP in diseases such as atypical hemolytic uremic syndrome (aHUS) (46) and C3 glomerulopathy (47). Nevertheless, several features of these AP-mediated diseases remain unexplained. Although a large number of complement defects has been identified in patients with aHUS, no predisposing conditions are identified in up to 40% of patients (12, 46). This suggests that other factors that are not traditionally regarded as part of the complement cascade can affect AP activation. It is also not clear why specific tissues are susceptible to AP-mediated injury in patients with systemic defects in AP regulation. Furthermore, disease flares are frequently triggered by infections (12) or tissue ischemia (48, 49). Finally, it is noteworthy that patients with active disease do not always respond to treatment with factor H–sufficient plasma, even if the primary risk for aHUS is a deficiency in factor H (12). These clinical observations indicate that local factors influence activation of the AP by factor H, and the results presented in this article provide a potential explanation for these findings.

The results in this study expand our general understanding of how the complement system is regulated. The ability of factor H to discriminate between host cells and invasive pathogens has been attributed to binding of the molecule to negatively charged molecules, such as sialic acid moieties and GAGs that are displayed on the surface of host cells (1). In diseases such as age-related macular degeneration and aHUS, the anatomic restriction of disease may be due to the tissue-specific distribution of these binding ligands or to differences among tissues in sulfation of the expressed GAGs (50). A set of proteins that is closely related to factor H, known as the factor H–related proteins (in humans designated factor H–related proteins 1–5), was recently identified as possible antagonists of FH (51–53). Each of these proteins possesses a degree of sequence identity with regions of CFH that are involved in GAG/sialic acid binding and with C3b binding and, additionally, may bind to other ligands recognized by factor H (28, 54–60). Our data demonstrate that regulation of the AP by factor H on specific surfaces is also affected by expression of annexin A2. Therefore, multiple proteins can likely dysregulate the AP through their interactions or competition with factor H. It is also likely that the balance of these molecules determines the overall AP activity on a given surface. The factor H–related proteins are primarily produced in the liver (61), but tissues can actively regulate their expression of annexin A2, thereby affecting local AP activity.

A limitation of our study is that we have not identified the region of factor H that interacts with annexin A2. A previous study reported that the SCR 6–8 region of factor H binds to annexin A2 (20). This region of factor H mediates binding to tissue surfaces, but it does not directly mediate complement regulation. Our results indicate that annexin A2 disrupts fluid-phase regulation (Fig. 4C, 4D) and cell surface complement regulation (Figs. 6, 7) by factor H. A direct interaction of annexin A2 with the first four SCRs could impair regulation in the fluid phase and on surfaces. It is also possible that binding of annexin A2 to other regions of the protein (e.g., SCR 6–8) changes the conformation of factor H, thereby affecting fluid-phase complement regulation by the protein.

Our data indicate that therapies that block the interaction between annexin A2 and factor H could ameliorate complement-mediated inflammation. Such a therapeutic strategy could theoretically reduce inflammation in patients with diseases associated with increased annexin A2 expression, but such an agent would have less effect in healthy individuals or in unaffected tissues. This approach would be preferable to untargeted immunomodulatory drugs that completely block immune functions throughout the body. However, based on our data, agents that interfere with the annexin A2–factor H interaction could still increase a patient’s risk for infection. In addition, annexin A2 influences hemostasis on the surface of endothelial cells (62), and biologic agents that target annexin A2 could be thrombophilic (62). Ideally, therapies that target the annexin A2–factor H interaction would not disrupt the other functions of annexin A2.

In conclusion, we found that the protein annexin A2 is upregulated in the kidney after I/R and binds to factor H. Annexin A2 reduces the binding of factor H to cell surfaces, increasing AP activation on the cells. This novel mechanism of complement activation can improve bacterial clearance by the AP, but it also increases complement activation on host tissue surfaces. In patients with infections or tissue ischemia, the increased production of proteins, such as annexin A2, that interfere with factor H function could trigger complement activation. Local production of these proteins could also explain why systemic defects in AP regulation frequently lead to tissue-specific disease. Therapies that block the annexin A2–factor H interaction may increase complement regulation by endogenous factor H and reduce tissue injury in AP-mediated diseases.

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**References**


