Review

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The possible role of factor H in complement activation-related pseudoallergy (CARPA): a failed attempt to correlate blood levels of FH with liposome-induced hypersensitivity reactions in patients with autoimmune disease

Abstract: Factor H (FH) is a natural inhibitor of the alternative pathway (AP) of complement (C) activation, an abundant protein in blood whose reduced level has been associated with proneness for increased C activation. There are also 5 FH-related proteins (FHR), which have different impacts on C function. After brief outlines of the C system and its activation via the AP, this review focuses on FH and FHR, collecting data from the literature that suggest that reduced levels or function of FH is associated with C activation-related hypersensitivity reactions (HSRs), called C activation related pseudoallergy (CARPA). Based on such observations we initiated the measurement of FH in the blood of patients with inflammatory bowel disease (IBD) and rheumatoid arthritis (RA), and examined the correlation between FH levels and HSRs following i.v. administration of PEGylated liposomal

prednisolone phosphate (PLPP). ELISA assay of FH was conducted on plasma samples before treatment, immediately after treatment and at follow-up visits up to 7 weeks, and an attempt was made to correlate the FH levels obtained with the presence or absence of HSR that occurred in five of twenty patients. However, the initial data presented here on three reactive and three non-reactive patients showed FH levels $>600 \mu\text{g/mL}$, while the normal range of FH is $2\text{--}300 \mu\text{g/mL}$. This unexpected outcome of the test led us to realize that the ELISA we used was based on antibodies raised against the short consensus repeats (SCR) in FH, which are also present in FHR. Thus the kit cannot distinguish these proteins and we most likely measured the combined levels of FH and FHR. These initial data highlighted an unforeseen technical problem in assessing FH function when using a FH ELISA that cross reacts with FHR, information that helps in further studies exploring the role of FH in CARPA.

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Introduction: A primer in complement and factor H

The complement (C) system

One of the major tasks of the C system is to mark and dispose of potentially dangerous particles such as

pathogenic microbes and altered host cells (1). This is achieved by the recognition of and targeted activation on foreign surfaces and modified host targets, such as apoptotic cells. The classical and lectin C pathways are activated upon recognition of certain molecular patterns associated with microbes or altered self, whereas the alternative pathway is activated constantly at a low rate and in an indiscriminative manner. Importantly, complement regulators protect the host from bystander damage.

Artificial surfaces such as stents, cannulae, nanoparticles, liposomes etc. represent foreign surfaces that may cause activation of the complement system. Complement activation can occur via the three above-mentioned pathways and may proceed, if not attenuated by regulators, to the initiation of the terminal pathway. The terminal pathway is activated upon the cleavage of C5, which generates the inflammatory mediator C5a, and C5b, which by binding additional components can result in the formation of terminal complement complexes (C5b-9 or TCC). C5b-9 when inserted in the target cell membrane forms pores (termed membrane attack complex; MAC) that result in lysis.

The alternative pathway of C activation

The internal thioester bond in C3 can undergo spontaneous hydrolysis, resulting in C3(H₂O), which is able to form the C3 converting enzyme C3(H₂O)Bb. This enzyme cleaves C3 molecules into C3a and C3b. C3a is an anaphylatoxin and C3b can bind covalently via its thioester group to nearby hydroxyl or amino groups thus C3b can deposit to target surfaces. In addition, C3b can form fluid phase or surface alternative pathway C3 convertase enzyme (C3bBb). Because each of the three complement pathways leads to the cleavage of C3 into C3b, the alternative pathway can amplify the cascade reaction started by any pathway (called the “amplification loop”). Thus, even if initial activation is due to the classical or lectin pathway, alternative pathway activity may be responsible for the majority of observed total complement activation (1, 2).

Because of this amplification function of the alternative pathway, its proper regulation in the host is particularly important in order to maintain cell and tissue integrity (2). Indeed, a number of complement regulatory proteins, both fluid-phase inhibitors and cell membrane bound proteins, act at the C3 level(3).

This also implies that therapeutic alternative pathway inhibitors, particularly those acting at C3/C3b, such as FH, can effectively down-regulate C3 fragment deposition and terminal pathway activation.

FH structure and function

FH is the main inhibitor of the alternative complement pathway [reviewed in (4, 5)] (Figure 1A). It is a 155-kDa plasma glycoprotein with a serum concentration of ~250 µg/mL. It is a cofactor for factor I in the enzymatic degradation of C3b and also inhibits the C3bBb convertase by preventing its assembly and, when C3bBb already formed, accelerating its decay. Factor H is composed of 20 short consensus repeat (SCR) domains (also termed complement control protein domains, CCPs) (Figure 1B).

The cofactor and decay accelerating activities reside in the N-terminal SCRs 1-4, which also represent one of the two main C3b binding sites within the molecule. The C-terminal SCRs 19-20 contain the second main C3b binding site and this site interacts with the TED domain (residing in the C3d fragment) of the C3b molecule. These domains also harbor a major binding site for cell surface polyanionic host markers, such as sialic acid and glycosaminoglycans. This allows factor H to bind to C3b/C3d deposited on host cell surfaces and thus to discriminate between self and

Figure 1: Complement activation and the role of factor H.

(A) Complement is activated via three major pathways, the classical, the lectin and the alternative pathway. Activation generates C3 converting enzymes that cleave C3 into C3a and C3b. C3b feeds into the alternative pathway, allowing an amplification loop of complement activation. C3b also binds to the C3 convertases which then are able to cleave C5 into inflammatory C5a and C5b, which by binding additional components can form lytic C5b-9. Factor H inhibits the AP by facilitating the cleavage of C3b by factor I and the inactivation of the AP C3 convertase. (B) Schematic structure of FH. FH is composed of 20 SCR domains. SCRs 1-4 mediate the complement regulatory functions of FH and SCRs 19-20 allow binding of FH to host cell and tissue surfaces. C3b binding sites are located in the same domains; in addition, glycosaminoglycan (GAG) binding sites are found in SCR7 and SCRs 19-20.

non-self (6). Thus, Factor H acts in the fluid phase (i.e., in plasma and other body fluids) and also inhibits complement activation on host surfaces, such as cells and basement membranes, where it can bind via its glycosaminoglycan/sialic acid binding site. In contrast, microbes normally do not express host-like polyanionic molecules and factor H cannot bind to them, allowing C3b deposition unchecked and progression of the activation cascade (4).

FH is the prototypical member of a family of related proteins, which include FH-like protein 1 (FHL-1), a 42-kDa serum glycoprotein derived via alternative splicing from the FH gene, and five FH-related proteins (FHR-1 to FHR-5) that derive from separate genes adjacent to the FH gene (Figure 2) [reviewed in (7)], FHL-1 includes the SCRs 1-7 of FH plus a four amino acid-long unique C-terminal end and possesses FH-like cofactor and convertase decay accelerating activities. In contrast to this, the five FHR proteins lack domains related to the SCRs 1-4 of FH and lack such activities. The FHRs have 4-9 SCR domains that exhibit various degrees of amino acid sequence identity to certain FH domains, ranging from distant homology (e.g., 36–42% of SCRs 1-2 of FHR-1, FHR-2 and FHR-5 to SCRs 6-7 of FH) to high similarity (e.g., 95–100% in the three C-terminal SCRs of FHR-1 to FH domains SCRs 18-20). Recent data

suggest that the FHR proteins can antagonize FH function by competing with FH for ligands such as C3b, thus these proteins seem to enhance complement activation through this mechanism termed de-regulation (8–10). Thus, the relative amounts of FH and FHRs as well as their affinities to common ligands and surfaces will determine the degree of AP C inhibition by FH locally. A further important consequence of the existence of these closely resembling proteins is that most antibodies, both monoclonal and polyclonal, raised against FH cross-react with one or more of the FHR proteins and FHL-1. Thus, simple immunostaining or ELISA detecting “FH” may be misleading if there is no precise information available on the cross-reactivity of the applied Abs.

FH deficiency in diseases

There are a number of pathological cases arising from FH dysfunction due to its central role in complement regulation (4, 11). Overactive FH leads to reduced complement activity against pathogenic agents, thus increasing the susceptibility to microbial infections. However, underactive FH can increase unwanted C activity against healthy host cells leading to complement-mediated disease. These pathologies usually are the results of mutations in the protein or polymorphism. As an example, patients with homozygous FH deficiency leading to underactive FH can suffer from recurrent bacterial infections like *Neisseria sp.*, furthermore vasculitis and glomerulonephritis. In studies of patients with hypocomplementemic glomerulonephritis heterozygous deficiency with mutations of conserved cysteine residues in SCR 9 and 16 were described. These mutations disrupt intrachain disulfide bridges, thereby perturbing the higher order structure of FH. This leads in turn to a profound selective block in secretion of the FH (12). On the other hand, recent studies showed hyperactivation of the alternative C pathway in neuropsychiatric disorders like schizophrenia: single nucleotide polymorphisms (SNPs) of gene encoding FH was found, CFH rs424535 (2783-526T>A) SNP was positively associated with schizophrenia. Moreover, SNPs rs800292(184G>A) was positively associated with stroke in ischemic stroke studies (13).

Figure 2: FH-like proteins.

(A) In humans, six genes on the long arm of chromosome 1 encode the members of the FH protein family. The CFH gene gives rise to FH and FHL-1 and five CFHR genes encode the FHR proteins. (B) The domain composition of the FH, FHL-1 and FHR proteins. FH is composed of 20 SCR (or CCP) domains. FHL-1 is identical to the N-terminal SCRs 1-7 of FH plus includes 4 aminoacids at its C terminus. The individual FHR proteins include four to nine SCRs, which are shown in vertical alignment to the homologous domains of FH. The similarity of homologous domains varies from 32 to 100% (not shown in the figure).

FH deficiency correlating with radiocontrast reactions

A thorough survey of the literature on the relationship between FH levels and HSRs in patients led to the group of

Phillip Lieberman et al., who published two papers focusing on HSRs to radiocontrast agents (14, 15). Radiocontrast agents are a type of medical contrast medium used to improve the visibility of internal bodily structures in X-ray based imaging techniques such as computed tomography (CT) and radiography. They analyzed the adverse reactions to metrizamide, iohexol, ioversol, iopamidol and concluded that next to penicillin these contrast materials are the most significant causes of anaphylactoid reactions worldwide. According to the quoted statistics, the above iodinated radiocontrast materials caused HSRs in approximately 5–9% of patients treated.

Importantly, among the risk factors that showed significant association with radiocontrast reactions in the studies of Lieberman et al., reduced FH levels or FH dysfunction were listed. Radiocontrast reactions, on the other hand, are known to be caused, at least in part, by C activation (16, 17). The two facts taken together suggest that reduced FH levels or FH dysfunction might sensitize people for not only radiocontrast agent-induced, but all drug-induced CARPA because of reduced natural suppression of C activation via the AP. Consequently, reduced FH levels can be used as a laboratory predictor of i.v. drug-induced CARPA.

FH – as predictor of liposome-induced HSRs

Based on the above theory on the potential use of FH as a biomarker for drug-induced CARPA, we recently carried out a study wherein the protein profile in the plasma of a

normal human subject was analyzed, who showed proneness for C activation by liposomal doxorubicin (Caelyx) in vitro (referred to as Caelyx-sensitive plasma, CSP) (18). The aim was to find one or more specific changes that could be considered as a biomarker for increased susceptibility for C activation. The proteome profiling was done with a library of human plasma proteome specific mAbs on chips (PlasmaScan-380TM) that have been printed on a microscope slide-sized glass plate (six/plates), each containing 380 different mAbs raised against non-redundant (with respect to epitopes). The analysis revealed 8 proteins that were differentially represented in CSP in comparison with Caelyx-insensitive control plasma (Table 1).

Among the significant changes we found that the level of FH decreased 1.9-fold in CSP, while the level of FHR was increased 3.4-fold (Table 1) (18). The decrease in FH was consistent with proneness for increased C activation, while the increase of FHR was difficult to interpret.

In another recent study Kuznetsova et al. showed the presence of FH, along with C3b, among the proteins bound in plasma to liposomes loaded with diglyceride conjugates of melphalan and methotrexate (Mlph-DOG and MTX-DOG). Interestingly, neither fragment C3 nor FH was detected in the protein “corona” in the absence or decreased MTX-DOG content, suggesting that the drug has significant impact on C protein deposition on liposomes. It was concluded that the liposome composition defines the surface properties, which in turn determines the set of plasma proteins bound and thus causes inertness or reactivity of liposomes in circulation (19).

The above information on an inverse correlation between FH levels and risk of HSR to radiocontrast reactions, the in vitro preliminary data on FH levels in a

Table 1: Protein representation changes in Caelyx sensitive relative to Caelyx insensitive plasmas.

Protein	Direction of change	Fold change: Caelyx vs. Caelyx insensitive plasmas		
FH	Down	−1.89±0.03	−2.25±0.68	−1.08±0.05
FH and fHRP	Up	3.42±0.41	1.88±1.35	1.03±0.71
Serum amyloid P	Up	4.40±1.33	1.78±1.59	7.53±5.97
Fibronectin	Up	1.37±0.48	3.23±0.75	1.35±0.61
Apolipoprotein B100	Up	1.33±0.12	1.35±0.15	1.66±0.38
Alpha-2-HS-glycoprotein	Up	1.66±0.45	2.21±1.98	2.54±1.62
C4A	Up	1.90±0.45	1.47±1.05	0.86±0.44
Protrombin	Up	1.49±1.09	2.37±1.10	1.01±0.89

Values in the table show the averaged global normalized intensity ratio of the different plasma samples (mean±SD). Bolded entries are ratios when the corresponding residuals were higher than $2 \times$ of SD of the calculated residual mean. The table shows the monoclonal antibody array-bound labeled proteins, whose raw and globally normalized pixel intensities were significantly lower (down) or higher (“up”) than those in (3 different) Caelyx insensitive plasma samples. These proteins repeatedly showed statistically significant deviation from the microarray’s inherent variations in at least two out of 3 similar hybridizations. Their identities were determined via immunoprecipitation using dynabeads, followed by MS and Western blot analyses, as described. Table reproduced from (18) with permission.

hypersensitive serum and the information on liposome content influencing FH binding to liposomes led us to analyze samples from the clinical study described below to further our understanding of the role of FH in HSRs to i.v. drugs, including liposomes.

Preliminary results of a clinical study testing the correlation between FH levels in the blood of PEGylated liposomal prednisolone phosphate-treated patients and rise of HSRs

Patients and Samples

Blood samples were collected from patients suffering from autoimmune diseases (inflammatory bowel disease and rheumatoid arthritis), who participated in two clinical trials wherein they were treated with PEGylated-liposomal prednisolone phosphate (PLPP) administered in infusion. All participants provided written informed consent. The clinical trials were approved by the local institutional review board and conducted according to the principles of the International Conference on Harmonisation–Good Clinical Practice guidelines (Clinicaltrials.gov registration NCT01039103, NCT01647685). In total 22 patients were treated with two injections of PLPP (150 mg) with a two-week interval. Plasma samples were taken before treatment, immediately after treatment and at times of their follow-up visits, initially weekly, later two-weekly. Samples were stored at -80°C until shipping to Hungary on dry ice for the FH assays, in the framework of collaboration. Further details and ultimate evaluation of the clinical study will be described elsewhere.

FH ELISA

The human FH ELISA is a standard immune assay using primary monoclonal and secondary (peroxidase bound) antibodies which recognize the SCR on the FH molecule (Figure 1). The HK342 Human Complement FH ELISA kits were obtained from TECOmedical (HK342 Human Complement FH ELISA kit, Hycult Biotech, Uden, The Netherlands). The assay was performed by following the manufacturer's instructions.

Clinical observations

Hypersensitivity reactions were seen in 5 out of 22 patients in these studies. Additionally, two other subjects experienced mild symptoms consistent with an infusion reaction which were not reported as such. Symptoms by which an infusion reaction could be identified were erythema, shortness of breath, itching, flushing and shivers. The infusion reactions were mostly reported as mild and resolved without sequelae after temporarily halting the infusion. In one case a fever was reported (39°C).

Plasma FH levels

After appropriate dilutions, all OD values in the test samples were within the specified effective dynamic range of the assay (5–120 ng/mL) (Figure 3), thus, the ELISA proved to work as expected.

Figure 4A and B show the FH levels in the blood of patients displaying or not displaying HSRs, respectively.

The values did not show any trend in initial levels or subsequent changes that would reflect a role in the rise of HSRs. However, it is important to point out that FH levels were in the 600–3000 $\mu\text{g/mL}$ range. According to literature, while previous studies reported plasma FH concentrations ranging 265–684 $\mu\text{g/mL}$ (20), recent studies using monoclonal antibodies reported mean FH concentrations of 233 $\mu\text{g/mL}$ and 269 $\mu\text{g/mL}$ (21). On the contrary, the FH ELISA kit used in the current study measured 400–800 $\mu\text{g/mL}$ normal range according to its manual. So even if we accept the higher normal range, in the group of patients with HSR, patient 23 had higher baseline (pre-infusion sample) value than the other two shown, and also the samples taken from this patient were abnormally

Figure 3: Standard curve of the Hycult FH ELISA kit.

Figure 4: FH levels in patients who did not display HSR during treatment with PLPP (A) and in those who got HSR (B). Samples were taken pre and post treatment, then at 4, 5, 6 and 7 weeks later. Factor H levels were measured in the samples as described in the methods. Color code: Red: Pre Inj., Purple: Post inj., Orange: week 4, blue: week 5, Black: week 6, Green: Week 7. Y values, representing FH concentrations, are given in $\mu\text{g/mL}$. The numbers above the charts are patient numbers.

higher than the other patients'. At the patient group with no HSR, patient 09 had a high baseline (pre-infusion), and patient 9 and 11 had abnormally high FH values (See Figure 4A and B). These observations can be most easily rationalized by the fact that the ELISA was not measuring only FH but also FHR, which also carry the SCR antigen for which the ELISA was developed. Alternatively, the high FH levels could be attributed to the autoimmune disease of the patients, although the major rise we observed would be unexpected on the basis that autoimmune diseases are often associated with FH deficiency (see earlier comments on FH diseases).

Discussion and outlook

Complement activation induced by CARPA-genic materials, such as liposomes and radiocontrast media, may be inhibited by engineered or natural C inhibitors. Therefore, it is plausible that levels of such natural inhibitors like FH in plasma of patients with HSRs can influence proneness to and severity of HSRs. Indeed, previous studies suggested that FH levels may be correlated with severity of HSRs (14, 15). However, determination of FH levels is

complicated by the presence of at least six other related proteins (FHL-1 and FHRs) in plasma/serum samples (4). Increased FHR levels could also result in less regulation by FH due to their antagonistic effect on FH function via competition with FH for certain ligands and surfaces. This may explain the association of increased FHR protein level in Caelyx sensitive plasma compared with Caelyx non-sensitive plasma (18).

The FH measurements presented here did not show any possible correlation between FH level changes and HSR occurrence, most likely because the FH ELISA kit measures not only FH but also FHR, which also carry the SCR antigen for which the ELISA was developed. For overcoming of this technical barrier, a truly FH specific ELISA would be needed that excludes any FH-related proteins in the measurements.

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Josbert Metselaar (Rotterdam, July 6th 1971) obtained a MSc degree in Pharmaceutical Sciences in 1995 and a PharmD (Doctor of Pharmacy) degree in 1998, both at Utrecht University. During his study he completed a research internship in pharmacology and PK/PD modeling at the Dept of Pharmaceutics, University of Florida, US. In 1999 he started his PhD at the Dept of Pharmaceutics and the Dept of Immunology Veterinary Medicine in Utrecht within the scope of a large academic research collaboration funded by the Japanese pharmaceutical company Yamanouchi (nowadays Astellas). He studied novel targeted formulations of anti-inflammatory compounds in autoimmune diseases. After completing his PhD in 2003 and a Post Doc fellowship in the same field of research in 2005, he decided to on translating his academic accomplishments into novel clinical and industrial investigational products. To this end he founded his own University spin off company Enceladus Pharmaceuticals, with which he raised significant funding over the years.

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Gert Storm is a (bio)pharmaceutical scientist at Utrecht University. He studied biology and obtained his PhD at the Dept. of Pharmaceutics of the same university. His research interests are in the fields of biopharmaceutics and drug targeting. In his early career, he worked at Liposome Technology Inc. (USA), at the San Francisco (UCSF) School of Pharmacy and at Pharma Bio-Research Consultancy B.V. (Netherlands). In September 1991 he took up his position at Utrecht University. In 1999, he was appointed adjunct professor at the Royal School of Pharmacy, Copenhagen, and since 2009 he has been Honorary Professor in Biomacromolecular Drug Delivery at the University of Copenhagen. In 2000 he was appointed as a professor

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Janos Szebeni is the head of the Nanomedicine Research and Education Center at Semmelweis Medical University, Budapest, Hungary. He is an internationally recognized expert in the field of complement research. He has published many research papers in the field and has given numerous presentations at various scientific forums. He also introduced the term Complement Activation Related Pseudoallergy (CARPA), and is editor of the book Complement: Novel Roles in Health and Disease. His work in the past has encompassed a range of topics, including liposome based blood substitutes, HIV pharmacotherapy, and bone marrow transplantation. He has spent most of his professional career in the United States at the National Institutes of Health and other research institutes.