Complement Activation-Related Pseudoallergy: Biophysical, Immunological and Physiological Studies to Explore the Mechanism

PhD Thesis

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1 Introduction

Nanotechnology is one of the industrial revolutions of our age and its subject is the exploration, shaping and manufacture of materials in the 10^{-9} to 10^{-6} m particle size range. Bulk materials within this range of particle size take on special physical properties. The term nanotechnology was coined by Taniguchi in 1974. Currently the volume of related research and industrial output includes hundreds of research institutes and companies, over 100.000 scientific papers and patents as well as a commercial turnover of several hundred millions Euros in 2015. The European Union issued a draft guidance covering the safety and ethics aspects of development and marketing of nanotechnology based products.

One of the fastest-evolving fields of nanotechnology is nanomedicine. Applications include diagnosis, therapy, disease prevention and control. In particular, nanotechnology is able to greatly improve physico-chemical properties of drugs such as solubility, enable superior pharmacokinetic properties, targeting, decrease side effects and introduce new applications (multifunctional drugs, theragnostics).

Nanomedical development faces its distinct challenges. One of the most important phenomena of clinical concern are hypersensitivity reactions (HSR) during intravenous (i.v.) administration of certain nanomedicines. These reactions are also known as ‘infusion’ or ‘anaphylactoid’ reactions. The potential severity of these reactions poses a barrier to the therapeutic use of many state-of-the-art nanomedicines such as liposomal and micellar drugs, or therapeutic antibodies. A common feature of these HSRs

are non-IgE-mediated and generally the manifestations and underlying mechanisms do not fit into the Type I hypersensitivity classification, therefore the category ‘pseudoallergy’ is usually invoked. Progress of these reactions includes activation of the complement (C) system, and the term Complement Activation-Related Pseudoallergy (CARPA) is applied to more specifically refer to these events.

2 Objectives

2.1 Aggregation of PEGylated liposomes driven by hydrophobic forces

Polyethylene glycol (PEG) is commonly used in drug delivery to modify pharmacokinetic properties of active agents. Attaching PEG chains may effectively reduce their enzymatic degradation and prolong their circulation time in blood. PEGylation of liposomes helps them to evade phagocytosis by macrophages and results in dose-independent pharmacokinetics.

Ammonium sulfate (AS) and other kosmotropic agents may elicit the aggregation and even the fusion of PEGylated liposomes. The aggregates are formed by hydrophobic interactions due to the solvophobic effect of increasing salt concentration. An understanding of the mechanism of aggregate formation could provide invaluable information for successful drug formulations, therefore, first, we examined mechanism of aggregation of PEGylated liposomes.

2.2 Role of anti-PEG IgM in PEGylated liposomes caused anaphylaxis

One of the most studied examples of CARPA is the HSRs to PEGylated liposomal doxorubicin (Doxil). Second aim was to elucidate the role of C in porcine HSRs, focusing on the reactions caused by Doxil. Specifically, we studied the involvement of anti-PEG antibodies in these reactions on the basis of three key information. First, these antibodies may play an important role in C activation via classical pathway. Second, C activation was shown to be a major contributor to the accelerated blood clearance (ABC phenomenon) of repeatedly administered PEGylated liposomes in mice and rats. Third, in case of Pegnivacogin withdrawal from the market the anaphylactic reactions were linked to high blood levels of anti-PEG antibodies.

2.3 Inhibition of complement activation by Factor H *in vitro*

The antifungal drug, liposomal Amphotericin-B (AmBisome) and Paclitaxel (Taxol), a widely used anticancer drug solubilized by Cremophor EL (CrEL) micelles, were shown to activate C partly via the alternative pathway (AP), and to induce CARPA in pigs. Various therapeutic antibodies also cause HSR. Thus, it was hypothesized that inhibition of AP C activation might interfere with the CARPA activity of these drugs. Therefore, our aim was to assess the capacity of the natural AP regulator FH and its engineered derivative mini-FH to inhibit C activation induced by AmBisome, CrEL and the therapeutic antibody Rituximab *in vitro*.

2.4 Involvement of complement activation in the pulmonary vasoactivity of polystyrene nanoparticles

A recently published study questioned the role of C in polystyrene nanoparticle (PS-NP)-induced HSRs in pigs. The study concluded that the phenomenon can be explained by a fast phagocytic response (RPR) wherein the C activation did not play a role. Accordingly, the „RPR theory" was proposed as a new theory of HSRs to compete with the CARPA concept. The aim of our experiments was to elucidate the role of C activation in the PS-NP-induced HSR in pigs.

3 Methods

3.1 Aggregation of PEGylated liposomes driven by hydrophobic forces

Composition of liposomal suspension similar to the FDA-approved and marketed Doxil was prepared with the extrusion method. The size distribution of liposomes and aggregates were characterized by dynamic light scattering (DLS) on a Malvern Zetasizer Nano S. The aggregation was followed by turbidimetry and DLS, while the surface-charge was followed by Zeta-potential measurement. Morphology of aggregations was recorded with atomic force - and phase contrast microscopy. The hydration level of the PEG layer was estimated via infrared spectroscopy. C activation caused by liposomes was measured by sheep red blood cell assay.

3.2 Role of anti-PEG IgM in PEGylated liposomes caused anaphylaxis

Composition of Doxebo (Doxil vehicle, without Doxorubicin) similar to Doxil was prepared with the extrusion method. The animal experiments were performed with mixed breed male Yorkshire/Hungarian White Landrace pigs. Pigs were immunized with i.v. injection of Doxebo (0.1 mg PL/kg) or Pegfilgrastim (0.174 mg/kg) or rather after Doxebo subsequent treatment with Doxil (0.1 mg PL/kg) or human equivalent dose of Doxil (HED: 6.4 mg PL/kg). The CARPA experiment typically involved Doxebo, followed by a similar dose of Doxil, then 10x dose of Doxil, and, finally, 0.1 mg/kg Zymosan. In case of immunized pigs with Pegfilgrastim were injected Pegfilgrastim, Doxebo and Zymosan. In the course of hemodynamic changes were recorded the pulmonary (PAP) and systemic arterial pressure (SAP). Before and during the reactions in determined timepoints blood was taken whereby the levels of anti-PEG antibodies (IgG and IgM) and sC5b-9 were measured by ELISA method.

3.3 Inhibition of complement activation by Factor H *in vitro*

C activation of liposomes (prepared with the extrusion method), AmBisome, Cremophor EL (CrEL) and Zymosan were determined by sC5b-9 in normal human sera (NHS) by ELISA method and the effects of H-factor (FH) and recombinant mini-FH to these activators. In case of Rituximab (Rituxan) inhibition effect of FH and mini-FH were determined by sC5b-9 in whole blood by ELISA method.

3.4 Involvement of complement activation in the pulmonary vasoactivity of polystyrene nanoparticles

Size distribution of PS-NP and control liposomes (prepared with the extrusion method) were determined by DLS method, while Zeta-potential was measured by Malvern Zetasizer Nano ZS. Morphology by transmission electron microscopy, while hydrophobicity was assessed by Rose Bengal adsorption. C activation *in vitro* in pig serum was assessed by Western blot analysis of C3 fragmentation (iC3b, C3d and C3dg) and FACS analysis of the deposition of C3b and formation of terminal C complex (C5b-9) on PS-NPs. PS-NPs (equal surface) caused C activation *in vitro* in NHS was measured by C3a, Bb, C4d and sC5b-9 markers by ELISA method. The animal experiments were performed with mixed breed male Yorkshire/Hungarian White Landrace pigs. The PS-NPs (surface area of different NPs were equal) and Zymosan were injected in the animals as bolus. The hemodynamic changes of reaction were recorded by PAP and SAP.

4 Results

4.1 Aggregation of PEGylated liposomes driven by hydrophobic forces

The liposome precipitation began upon reaching an AS concentration threshold (0.7–0.8 M). PEGylated liposomes may not only have aggregated but also fused upon AS addition. The rate of fusion was concentration dependent, and the threshold concentration was 1 M at the employed incubation time. To disrupt aggregated liposomes they should be diluted well below the AS concentration at

which they started to precipitate. The liposome precipitation was not depended on surface-charge properties. Adding AS led to a massive increase of the Zeta-potential value at even relatively low concentrations (<0.1M AS). No precipitation took place in the absence of the PEG brush on the liposomal surface, while increasing the PEG coverage (from 2 to 10 M%), provoked precipitation in lower concentrations of AS. AS and other kosmotropic salts led to precipitation and the surface charge modifying effect of kosmotropic salts leveled off at much lower concentrations than that needed for aggregation. There were significant different between liposomes which were precipitated in 1 or 2 M of AS and after were reconstituted in physiological saline. Liposomes caused significant difference in C activation compared to control and also each other.

4.2 Role of anti-PEG IgM in PEGylated liposomes caused anaphylaxis

Immunization of pigs with Doxebo led to rise of anti-PEG antibodies (IgG and IgM) on day 3 following the treatment and reached peak between days 7-9 and returned to close to baseline after 4-6 weeks furthermore titers of antibodies changed at same rate. The anti-PEG IgM levels were not zero at baseline (time 0), indicating the presence of pre-existing (natural) anti-PEG antibodies. After Doxebo treatment the titers of natural antibodies dropped at day 1 compared to baseline.

Bolus injection of Doxil in not immunized pigs led to a moderate (2-fold) rise of PAP within 2 minutes, and the values returned to baseline within 10 minutes. The drop of anti-PEG IgM titer could be modeled by two phase exponential decay which is a

sum of a faster and slower ($T_{1/2}$ of ~3 minutes) process. It could be supported by the linear correlation (R^2=0.966) of PAP and anti-PEG IgM level changes calculated by the 1 minute values as baselines.

Pigs immunized with Doxebo showed severe reaction after i.v. injections of Doxebo/Doxil 4 weeks later, which the increased level of anti-PEG IgM caused (the dose-effect relationship between anti-PEG IgM and HSR reached plateau at a relatively low anti-PEG IgM level). In immunized pigs the injections of Doxebo/Doxil led to prompt rise of sC5b-9 (C activation) and with kinetics on the minute scale identical to that seen with PAP (anaphylatoxin production). In immunized pigs the first injection with Doxebo followed by second similar dose of Doxil caused essentially identical PAP and sC5b-9 changes.

In immunized pigs with Doxebo and Doxil HED the pretreatment prevented the anaphylactic response to liposomes 4 weeks later, and even the total HED of Doxil could be safely infused into the animal.

In Pegfilgrastim immunized pigs Pegfilgrastim induced a rise of anti-PEG IgM at week 1 post-immunization, at which time Pegfilgrastim did not induce HSR, but the hemodynamic reaction to Doxebo was amplified relative to that seen in naïve animals.

4.3 Inhibition of complement activation by Factor H *in vitro*

Exogen FH inhibited the effect of C activation of AmBisome, liposomes of various PEG-compositions and CrEL. Mini-FH also inhibited C activation of AmBisome and CrEL, however its effect was more pronounced in comparison with FH. Inhibitory effect of mini-FH was dose-dependent, the extent of inhibition suggested that

mini-FH is on a molar basis approximately twofold more potent inhibitor of C activation under these conditions than FH.

Rituximab caused C activation in anticoagulated whole blood, more so when Lepirudin was used as anticoagulant, compared to Heparin. Exogen FH inhibited C activation induced by Rituximab, however mini-FH strongly inhibited Rituximab-induced C activation in Lepirudin anticoagulated whole blood.

4.4 Involvement of complement activation in the pulmonary vasoactivity of polystyrene nanoparticles

All PS-NP dispersions, as well as liposomes, had a unimodal, relatively narrow size distribution. PS-NPs had a strong negative charge both at low ionic strength and at higher ionic strength. The hydrophobicity depended on NP size, with absolute values increasing in the order 200<500<750 nm diameter.

The FACS histograms illustrated the binding of C5b-9 and iC3b to 500 and 750 nm PS-NPs after 1 and 20 min incubation. Except for C5b-9 binding to 500 nm PS-NPs, such increase was observed in 4 of 7 pigs' sera for both labels. The maximal changes compared to baseline were significant ($P<0.05$). The delay of maximal staining of iC3b versus C5b-9 in individual pig serum was consistent with the kinetics of the formation of these C activation byproducts. 750 nm PS-NPs displayed stronger expression of these C activation markers than 500 nm NPs

In case of 500 and 750 nm NPs Western blot results indicated the formation of C3dg and C3d within 2-30 min. This was more pronounced with C3dg in case of 500 nm NPs and C3d in case of 750 nm ones.

500 and 750 nm spherical PS-NPs caused significant rises of sC5b-9 relative to PBS control in NHS, while 200 nm particles had only a minor, insignificant effect, or rather trend. 500 and 750 nm PS-NPs caused similar significant elevation of C3a and Bb like sC5b-9, while C4d did not change. Time course of changes of C3a showed that C activation might have started instantly, within minutes. sC5b-9 formation of 3 different doses (24.2, 72.7 and 218.1 cm^2/mL sera) of 500 nm NP showed near maximal effect at the latter dose in 2 of 3 tested sera.

The smallest dose of 200 nm NPs did not cause any change in PAP, while identical surfaces of 500 and 750 nm PS-NPs led to massive pulmonary hypertension. Although 10- and 40-fold higher doses of all 3 NPs caused major pulmonary pressure changes independent of dose, so the dynamic window of the pulmonary hypertensive effect of PS-NPs was in a NP size-dependent subthreshold dose range: <50 ng/kg (200 nm), <12,5 ng/kg (500 nm) and <19 ng/kg (750 nm), respectively.

The maximal PAP rises after injection of equi-surface doses of different PS-NPs in 5 pigs, vis-à-vis the rise of sC5b-9 caused by equi-surface doses (72.5 cm^2/mL serum) of the same PS-NPs in NHS. Between the paired mean sC5b-9 and PAP values were significant correlation (P=0.033).

PS-NP-induced pulmonary reaction of pigs was particle size-dependence. The minimally reactive NP number 10-fold led to differential increase of pulmonary reaction to differently sized NPs (200<500<750 nm). In contrast, increasing the minimal dose 40-fold yielded maximal effect only with the large (500 and 750 nm) PS-NPs.

5 Conclusion

5.1 Aggregation of PEGylated liposomes driven by hydrophobic forces

Our results attest that the aggregation of PEGylated liposomes was regulated and reversible manner, which was a concentration-dependent mechanism. Reversibility of aggregation was the simple consequence of the reduction of concentration of the kosmotropic agent. PEGylated liposomes may not only have aggregated but also fused upon AS addition. The rate of fusion was concentration- and time-dependent which may be explained by excessive structural alterations of PEG chains due to their hydrophobic modification.

Reduction of surface charge did not have any effect on aggregation of vesicles while the propensity for precipitation increased with PEG coverage. This clearly showed that AS-driven aggregation of PEGylated liposomes was related to the PEG chains.

Since, other kosmotropic salts also precipitated PEGylated liposomes, while chaotropic salts did not, this suggested, that the kosmotropic salt reduced the hydration of the PEG polymer chains in the course of the mechanism of aggregation.

According to result of sheep red blood cell assay sample which contained more fused vesicules caused higher C activation which explained the bigger size and thus increased surface where the C proteins deposited more efficiently.

5.2 Role of anti-PEG IgM in PEGylated liposomes caused anaphylaxis

The results obtained direct evidence of C activation via IgM-mediated classical pathway and proceeding in remarkable parallelism with the development of anaphylactic shock. The level of anti-PEG antibodies (IgG and IgM) increased same kinetic after pigs were immunized with Doxebo, which implied T-cell independent type-2 (TI-2) immunogenicity. After Doxebo treatment the titers of natural antibodies dropped at day 1 compared to baseline, which suggested liposome-induced clearance.

IgM-mediated classical pathway C activation, the identical time courses of initial rise of PAP and consumption of anti-PEG IgM and correlation between values of first minute of PAP and titer suggested that, at least in part, to the immediate binding of preformed (natural) anti-PEG IgM to the vesicles, led to C activation with anaphylatoxin production and opsonization in not immunized animals. Taken together with IgM-mediated ABC of PEGylated liposomes in murine models suggested that C activation and cardiopulmonary reaction coincided with rapid phagocytic clearance of the portion of Doxil that binds IgM. The indication of liposome excess over the antibodies resulted in near complete removal of anti-PEG IgM to a minimal baseline, explained the tachyphylactic. In immunized pigs the first injection with Doxebo was followed by essentially identical PAP and sC5b-9 changes to a second, similar dose of Doxil, attesting to the lack of Doxebo-induced tachyphylaxis.

Pretreatment of pigs with HED of Doxil inhibited the immunogenicity and pulmonary reactivity of liposomes. Since the titer of anti-PEG IgM did not change after pretreatment, it seems that the phenomenon related to suppression of PEGylated liposome-induced IgM production.

Pegfilgrastim induced a rise of anti-PEG IgM at week 1 post-Pegfilgrastim immunization, at which time did not induce HSR. The reason that, Pegfilgrastim was too small to provide appropriate surface for C activation.

5.3 Inhibition of complement activation by Factor H *in vitro*

Exogen FH and mini-FH efficiently inhibited AmBisome, liposome, CrEL and Rituximab induced C activation *in vitro*. The reason that FH is a major inhibitor at the level of the central C3 C component and that of the alternative pathway amplification loop, it is an ideal regulator to reduce or even prevent generation of the C3a and C5a anaphylatoxins, and also that of C5b-9, particularly, because C activation by any pathway would be amplified through this loop.

Mini-FH was more potent inhibitor than FH. The enhanced activity of mini-FH is likely due to the increased availability of the C-terminal glycosaminoglycan/C3d binding sites in the shorter molecules, which are probably partly hidden in the full-length FH.

The amount and nature of deposited C3 fragments, as well as the physicochemical properties of the surfaces, also influenced the potential interaction of FH with these surfaces. These at least in part explained why the same amount of tested FH resulted in different degree of inhibition.

Exogen FH and mini-FH were able to reduce or inhibit Rituximab-induced C activation in serum *in vitro*. This is most likely due to fluid-phase inhibition of C activation by FH and mini-FH.

5.4 Involvement of complement activation in the pulmonary vasoactivity of polystyrene nanoparticles

PS-NP showed differences from control liposomes in physicochemical features (size distribution, surface charge and hydrophobicity).

C3b/iC3b and C5b-9 deposition on PS-NPs on the same minute scale that the HSRs occur, proceeding in parallel with C3 fragmentation and formation of iC3b degradation products (C3d and C3dg) provided evidence for instant C activation and rapid opsonization of 500 and 750 nm PS-NPs in course of presence of HSR.

Same rise of level of Bb, C3a and sC5b-9 indicated 500 and 750 nm PS-NPs induced alternative pathway C activation. There was significant impact of NP size, and thus, surface geometry (curvature) in C activation which is likely to be inversely proportional to geometry (curvature) of particle. The reason that the efficiency of the buildup of C3/C5 convertases and opsonins are higher on flat than on curved surfaces.

6 Publications of the candidate

6.1 Publications related to the thesis

1) *Bozó T, ***Mészáros T**, Mihály J, Bóta A, Kellermayer MSZ, Szebeni J, Kálmán B. (2016) Aggregation of PEGylated liposomes driven by hydrophobic forces. Colloids Surf B Biointerfaces, 147: 467-474. IF: 3,887
2) **Mészáros T**, Csincsi ÁI, Uzonyi B, Hebecker M, Fülöp TG, Erdei A, Szebeni J, Józsi M. (2016) Factor H inhibits complement activation induced by liposomal and micellar drugs and the therapeutic antibody rituximab in vitro. Nanomedicine,12(4): 1023-1031. IF: 5,720
3) ***Mészáros** T, *Kozma GT, Shimizu T, Miyahara K, Turjeman K, Ishida T, Barenholz Y, Urbanics R, Szebeni J. (2018) Involvement of complement activation in the pulmonary vasoactivity of polystyrene nanoparticles in pigs: Unique surface properties underlying alternative pathway activation and instant opsonization. Int J Nanomed. 13: 6345-6357. IF: 4,370 (2017)

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6.2 Publications not related to the thesis

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