

Role of the complement system in nanoparticle-related pathologic immune processes

Doctoral theses

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Introduction

Since the amount of nanoparticles is rapidly increasing in the environment, it is ever more important to deal with the health effects of nanoparticles. Ultrafine particles can enter indirectly into the human body by inhalation of air. Among ultrafine particles, the 10-100nm and the 1-10 μ m sized particles can easily be deposited in the small airways. Particles bigger than 10 μ m are normally filtered from the inhaled air in the upper airways, but in the case of very high concentrations they can pass into the lungs. Microparticles involved in the pathogenesis of asthma are typically bigger than 10 μ m, for example pollen grains, house dust mite, dog- and cat allergens and mould spores. COPD related nanoparticles have 10-100nm size, such as tobacco smoke, asbestos grains and carbon-based toner ink. Long term accumulation of these nano- and microparticles can lead to chronic airway inflammation, which can also involve the complement system. While the main targets of inflammation are the bronchi in asthma, showing increased smooth muscle cell proliferation and neovascularization, deeper parts of the lung, the alveoli are deteriorated in COPD.

Nanoparticles can also be administered directly into the human body by the use of intravenous nanomedicines. In these cases

there is an increased risk for undesired immune reactions due to similarity with pathogens in size and other physicochemical features. Occasional development of complement activation related pseudoallergy (CARPA) has been described upon use of nanomedicines containing micelles, liposomes and microbubbles. During CARPA reactions, the nanomedicine structures activate the complement proteins in blood, and the generated C3a and C5a molecules stimulate the basophil granulocytes through C3aR and C5aR receptors. As effects of cell activation, histamin, triptase and other proteases are liberated from basophil granules, and lipid mediators (leukotrienes, prostaglandines, tromboxan, PAF) are cleaved from the cell membrane. These biologically active products are responsible for the development of anaphylactoid symptoms by causing bronchoconstriction, and by influencing blood vessel permeability and blood pressure.

During IgE mediated (true type) allergy, the same mediators are created due to the activation of eosinophil granulocytes and mast cells through their FcεR receptors. IgE mediated allergy is an important element in the pathogenesis of asthma, and causes the well known asthmatic bronchoconstrictions. The alternative pathway of the complement system is considered to play an important role in the development of bronchial hyperreactivity in asthma. Factor H, the regulator of

the alternative pathway has been shown to be downregulated in a proteomic analysis of rapid decliners in COPD.

Aims

Nanoparticles entering into the human body can cause acute non-IgE mediated allergic reactions in the blood, while nano- and microparticles inhaled and deposited in the lungs can maintain chronic inflammation in the body. In both cases, the complement system is presumed to be involved. Therefore we have marked out the following aims:

1. complement mediated pseudoallergy:

- 1.1. Screening for in vitro complement activation by intravenous drugs that are able to induce hypersensitivity reactions.
- 1.2. Identification of complement activation-inducing components in drugs with positive screening results.
- 1.3. Examination of the mechanism of complement activation in the case of complement reactive drugs.
- 1.4. Studying the clinical relevance of drug induced complement activation in the development and grade of infusion reactions.

2. *obstructive lung diseases based on chronic inflammation:*

- 2.1. Comparison of complement activation in blood and airway samples in asthma and COPD.
- 2.2. Comparison of H factor levels in blood and airway samples in asthma and COPD.
- 2.3. Examination of the association of H factor and SC5b-9 levels with airway obstruction and disease severity in asthma and COPD.

Methods

For the in vitro tests modelling CARPA reactions, sera were collected from 140 healthy volunteers, and the samples were randomly selected to the drug tests. Sizes of the drugs were determined by using a Zetasizer S (Malvern Ltd) instrument. In the in vitro drug tests, the drugs were incubated with the selected sera at 37°C, and complement activation was stopped after the incubation time by adding EDTA. Thereafter C3a, C5a, SC5b-9 and Bb concentrations were determined by using commercial ELISA kits (Quidel Ltd), and the concentrations were compared with the complement concentrations of untreated sera. Since the distribution of concentrations was Gaussian, we looked for significant elevations in complement levels with a paired t-test, or an ANOVA analysis combined with Bonferroni post hoc test.

In the CARPA clinical study, we collected baseline serum and plasma samples from 29 cancer patients before starting chemotherapy. During the chemotherapeutic infusions, we monitored signs for anaphylactoid reactions with the help of an ICG-NHMS cardiograph (ASKIT Ltd). In vitro complement activation tests were performed as described above by using the pretreatment sera and the drug assigned to chemotherapy, and the results were compared with the

presence and grade of hypersensitivity symptoms. ROC analysis was performed to determine the predictive value of pretreatment complement activation tests in the development of anaphylactoid infusion reactions.

For the asthma and COPD studies, 26 stable asthmatics, 17 stable COPD patients and 21 healthy volunteers were enrolled. Plasma and sputum samples were collected, lung function and fractional exhaled NO (FENO) were measured in each cohort. Actual control state of asthmatics was measured by ACT questionnaire, while CAT questionnaire was used to assess the state of COPD patients. SC5b-9 and factor H concentrations were determined in plasma and sputum samples by ELISA method (SC5b-9: Quidel, factor H: Hycult). Since the sputum concentrations showed non-Gaussian distribution, concentrations of different cohorts were compared by using Mann-Whitney and Kruskal-Wallis tests. The relationship between complement concentrations and clinical parameters of the patients was examined by Spearman correlation analysis.

Results

We compared the complement activating capability of AmBisome and Caelyx, and found that AmBisome caused complement activation in every studied sera, while the

proportion of Caelyx-reactive donors was 0,7%. AmBisome induced Bb generation in every observed sera, and Bb concentrations changed proportionally to SC5b-9 formation.

15 chemotherapeutics, which were different in size, were screened for complement activation-inducing ability. The results showed increased SC5b-9 formation after treatment with micellar drugs, Taxol and Taxotere in 25% and 40% of the treated sera, respectively. The small molecules platins, Gemzar, Vinblastin, Doxorubicin, Fluorouracil, Endoxan, Mitomycin and Irinotecan did not have any effect on complement activation, similarly to the two monoclonal antibodies (Erbitux and Herceptin). We showed for the first time that the complement activating effect of Taxotere is mediated by its micelle-forming compound, Tween 80. Both solvents of Taxol and Taxotere, the Cremophor EL and the Tween 80 induced C3a and C5a production, while we were not able to show Bb generation in the treated sera. We could reproduce the complement-activating effect of Tween 80 and Cremophor EL in fresh (unfrozen) serum and plasma samples. When we performed complement activation assay on sera filtered for 100, 300 and 1000kDa, we could not see any complement activation even in the 1000kDa filtered serum after incubation with Cremophor EL or Tween 80. In contrast, zymosan could not have an impact on complement activation

only in the 100kDa filtered serum. Taxol and Taxotere induced C3a, C5a and SC5b-9 production in the pretreatment sera of cancer patients, similarly to the sera of healthy volunteers.

Comparing asthmatic and COPD airway and blood samples, levels of products of complement activation were found to be higher in COPD than in healthy subjects and asthmatics. Elevated SC5b-9 concentrations were found in severe asthmatics compared to mild-moderate asthma patients.

Factor H levels showed a different pattern: we found no differences in plasma samples, while factor H levels were elevated both in asthma and COPD patients compared to healthy subjects. Significantly higher factor H levels were shown in the airway samples of the severe asthma group than in mild-to-moderate asthmatics and in healthy subjects. The airway factor H levels were in positive correlation to asthma severity, medication step and sputum eosinophil count, and were in negative correlation with asthma control and lung function parameters.

Conclusions

We showed that the infusion drugs AmbiSome, Caelyx, Cremophor EL and Tween 80 are able to induce complement

activation in human blood. Differences in the rate of personal susceptibility may indicate that there may be differences in the mechanism of CARPA reactions induced by the different drugs. Mechanical differences in the activation process are also assumable by the observation that the examined liposomal drugs were able to induce the alternative pathway, while the two micellar drugs were not able to induce it. We could prove that C3a and C5a (crucial elements of the CARPA hypothesis) are really generated in response to taxanes in the blood of both healthy and cancer donors, so anaphylatoxins can be responsible for the development of anaphylactoid symptoms. Biological relevance is supported by our results showing dose- and time-dependence of complement activation, and unchanged complement reactivity in freshly separated unfrozen sera and plasma.

While the nanoparticle-sized drugs can induce hypersensitivity reactions in blood, the inhaled nano- and microparticles can induce chronic inflammation and persistent or recurrent allergic reactions in the lungs, provoking asthma or COPD on the long term. These processes can generate activation of the complement system, which are more intensive in the lungs than in the blood according to our own results in terms of factor H levels. Bronchial factor H levels have been shown to

be associated with severity and airway obstruction positively in asthma. Further studies are needed to determine the role of complement factor H and complement activation in the pathomechanism of asthma and COPD, and to determine the usefulness of factor H as a biomarker. Complement factor H might be useful as biomarker inter alia in differentiating severe asthma and COPD, or in identifying different asthma phenotypes.

List of own publications

Own publications summarized in the doctoral thesis:

1. Szebeni J, Bedócs P, Rozsnyay Z, Weiszhar Z, Urbanics R, Rosivall L, Cohen R, Garbuzenko O, Báthori G, Tóth M, Bünger R, Barenholz Y. (2012) Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome. *Nanomedicine*, 8(2):176-184.
Impact factor: 6.692*
2. Weiszhar Z, Czúcz J, Révész C, Rosivall L, Szebeni J, Rozsnyay Z. (2012) Complement activation by polyethoxylated pharmaceutical surfactants: Cremophor-EL, Tween-80 and Tween-20. *Eur J Pharm Sci*, 45(4):492-8. **Impact factor: 3.212***
3. Weiszhar Z, A. Bikov, G. Galffy, L. Tamasi, I. Ungvari, C. Szalai, G. Losonczy, I. Horvath. (2013) Elevated complement factor H levels in asthmatic sputa. *J Clin Immunol*, 33 (2):496-505. DOI: 10.1007/s10875-012-9807-8. **Impact factor: 3.077***

*: predicted value based on impact factor of 2011.

Own publications not connected with the doctoral thesis:

1. A Bikov, A Bohacs, N Eszes; Weiszhár Z; I Ivancso; V Muller; J Rigo Jr.; Gy Losonczy; L Tamasi; I Horvath. (2012) Circulating and exhaled vascular endothelial growth factor in asthmatic pregnancy. *Biomarkers*, 17(7):648-54. **Impact factor: 2.215***
2. Makó V, Czúcz J, Weiszhár Z, Kiszél P, Herczenik E, Matkó J, Prohászka Z, Cervenak L. (2010) Proinflammatory activation pattern of human umbilical vein endothelial cells induced by IL-1, TNF and LPS. *Cytometry Part A*, 77(10):962-70. **Impact factor: 3,749**
3. Gergely P Jr, Blazsek A, Weiszhár Z, Pazar B, Poor G. (2006) Lack of genetic association of the Toll-like receptor 4 (TLR4) Asp299Gly and Thr399Ile polymorphisms with spondylarthropathies in a Hungarian population. *Rheumatology (Oxford)*, (10):1194-6. **Impact factor: 4,052**
4. É Morschl, I Bretus, I Pávó, L Topa, Weiszhár Z, F László. (2000) Nitric oxide-mediated mucus hypersecretion protects the stomach of ovariectomized rats. *Eur J Pharmacol*, 392(1-2): R5-R7. **Impact factor: 2,236**