Novel observations in the pathogenesis of dermatitis herpetiformis

PhD thesis

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Introduction

More than hundred and thirty years have passed since Louis Adolphus Duhring (University of Pennsylvania, Philadelphia, USA) published skin disease with blister formation called dermatitis herpetiformis (DH) (probably with other blistering disorders). Later, Louis-Anne-Jean Brocq (Hôpital Sant-Louis, Paris, France) described that it differs to classic blistering diseases (real vesicles can be observed rarely), therefore it was called Duhring-Brocq or Brocq–Duhring disease. The valid verification and diagnosis of DH is possible since the date of identification of the immunofluorescence diagnostic characteristics (Van der Meer, 1969). Thanks to intensive researches, we know that DH is an environmental antigen induced, a so-called gluten sensitive autoimmune disease associated with latent celiac disease. Over the past decades next to the transglutaminase (TG) 2 antibodies, the detection of TG3 antibodies was a great advance in understanding the disease, although some of the features are still difficult to understand.

Therefore, our research group's aim was to further investigate the pathomechanism of the disease. The dissertation presents the results of circulating transglutaminase 3-immonoglobulin A (TG3-IgA) immune complexes and the fibrinolytic potential and fibrin structure examinations.

The background of the immune complex theory in dermatitis herpetiformis

Although it has been assumed for a long time and several data indicate the role of immune complexes in DH, circulating immune complexes and with the new data TG3-IgA immune complexes were never identified. In spite of classical autoimmune blistering diseases, where the antigen of the skin deposited and the circulating antibodies are the same, in DH the IgA antibodies do not bind to the structure of the papillary dermis, which suggest, that the dermal IgA-TG3 aggregates are immune complexes. IgA deposits or "DH bodies" in the papillary dermis represented amorphous electron-dense granules ultrastructurally along the extracellular matrix. With double immunofluorescence the IgA is co-localized with TG3 in the papillary dermis and in the dermal vessel walls.

Renal IgA immune complexes were detected in kidney biopsy of some symptom-free DH patients without impaired kidney function and histological proven nephropathy. IgA nephropathy was rarely reported in DH. TG3 is present in the uppermost epidermis, in the

corneal and granular layer of the healthy epidermis, TG3 were not detected in the normal papillary dermis. The epidermal origin of papillary TG3-IgA deposits is uncertain. All this supports the hypothesis that circulating immune complexes are present in DH, and they can be trapped in the microcirculatory system of different organs. It was not detected in the small intestine, but it is expressed in various tissues, so other extracutaneous origin can be possible.

The fibrinogen-fibrin-fibrinolysis pathology in DH

I was confirmed several decades earlier, that extravascular deposition of fibrinogen, fibrin and fibronectin appears also with IgA in the papillary dermis.

It was also detected recently, that TG3 preserves it's activity within the cutaneous IgA-TG3 complexes, binds fibrinogen, which localization is the same with the TG3-IgA complexes, and shows a similar intensity of staining.

The clinical characteristic of the disease is that in some patient acral leukocytoclast vasculitis-like petechiae and purpuras appear mostly on fingers or toes.

We observed recently that untreated DH patients have an unexpectedly high prevalence of cryofibrinogenemia in plasma, while under GFD and/or dapson treatment it significantly decreases or disappears. It has been shown previously that dapsone treatment seems to decrease the amount of cryofibrinogen in vitro, but the exact mechanism of action is unknown. DH skin lesions had been initiated in vivo by autologous serum injection, but this reaction did not develop in response to plasma treated with heparin or an antifibrinolytic agent ε-aminocaproic acid. Some reports evidence the efficiency of heparin in the treatment of severe DH patients, who did not tolerate sulfones, but the exact mechanism behind this therapeutic effect has not been fully explored. The presence of plasma cryofibrinogen is indicating a temperature dependent pathology associated with the function of circulating fibrinogen. On the basis of all these findings, we assumed that fibrinogen-fibrin-fibrinolysis system also plays a role in the pathomechanism of DH. Our aim was to study the functional examination of fibrinogen, which has been also examined, while plasma can be the source of fibrinogen precipitating with the papillary IgA-TG3 immune complexes in DH.

Purpose

1. Based on the clinical and immunological features we assumed that DH is an immune complex disease. Our aim was to detect circulating TG3-IgA immune complexes with a newly developed sandwich ELISA method.

2. We evaluated the correlation between the circulating TG3-IgA immune complexes and TG3 IgA antibodies. Our aim was to examine the gluten dependency of circulating immune complexes. We investigated the immune complex values of untreated DH patients and under GFD retrospectively.

3. Based on the findings detailed in the introduction we assume the pathology of the fibrinogen-fibrin-fibrinolysis system in DH. Our aim was to study the capacity of the fibrin formation and fibrinolysis of DH patients.

4. Our aim was to investigate the fibrin structure in DH. We examined whether there was any association between the possible fibrinolytic difference and the alterations of fibrin structure.

5. We examined the effect of dapsone, a symptomatic treatment in DH, on the parameters of fibrinolysis or fibrin structure in vitro.

Methods

The diagnosis of DH

The diagnosis of DH was based on the clinical symptoms, routine skin histology and direct immunofluorescence (DIF), IgA, IgG type TG2 enzyme-linked immunosorbent assay (ELISA) and/ or the endomysial AB (EMA) tests. In all DH patients the IgA type TG3 ELISA was also performed.

Histological and immunofluorescence (IF) studies

Routine histological examination was performed at the Histology laboratory of Semmelweis University Department of Dermatology, Venereology and Dermatooncology and the IF examinations at the IF laboratory. EMA were measured by indirect immunofluorescence (ImmuGlo IMMCO Diagnostics, Buffalo, NY).

Anti-TG2 and TG3 IgA ELISA

TG2 IgA antibodies (Orgentec Diagnostika, Mainz, Germany) and the TG3 IgA antibodies (Immundiagnostik, Bensheim, Germany) were tested at the Central laboratory of Semmelweis University and the Genetic laboratory of our Department.

ELISA for the circulating TG3 – IgA immune complexes

Patients and methods

35 untreated patients with DH and 40 controls were investigated; 16 healthy subjects, 12 patients with pemphigus vulgaris (PV), and 12 patients with systemic lupus erythematosus (SLE).

1. The comparative study of the serum and plasma samples

   Plasma as well as serum samples of 8 untreated DH patients and 7 healthy subjects archived at the same time were compared.

2. The assessment of the BSA blocking

Sera of 6 untreated DH patients and 6 healthy subjects were compared parallel with and without BSA blocking.

3. The assessment of gluten-free diet

10 additional untreated patients with DH were also examined under gluten-free diet (GFD) and in remission (the mean duration of GFD was 5,8 years).

Circulating TG3-IgA immune complex ELISA

Circulating TG3-IgA immune complexes were measured by a newly developed sandwich ELISA method, the method was published and detailed in the thesis. Wells were coated with polyclonal goat anti-human TG3 AB (Zone et al., 2011) and detected with anti-human IgA conjugate. Plasma/serum samples were tested in triplicate. The absorbance value indicated the level of circulating TG3-IgA immune complexes measured by ELISA.

Statistical procedures

Statistical analyses were performed with Statistica 8.0 (Statsoft Inc, Tulsa, OK). Departure from normal distribution assumption was tested by the Shapiro-Wilk test. Values were reported in median with the corresponding interquartile range [IQR]. Mann–Whitney U test, Wilcoxon test and Kruskal-Wallis test were used for group comparisons. Multiple comparisons of mean ranks for all groups and Tukey test were applied for post hoc analysis. P value of less than 0.05 was considered statistically significant.

The analysis of fibrinolysis and the structure of fibrin

Patients and methods

Twenty-three DH patients and 12 healthy controls were enrolled in the turbidimetric clot-lysis assay study. We measured the t-PA induced lysis time, (indicator of the global fibrinolytic activity) and the maximal turbidity (indicator of the fiber size of fibrin) by spectrophotometer at 340 nm at 37 ^{o}C. Controls were healthy adults, who did not take any drug affecting

coagulation. Out of the total 23 DH patients the following subgroups were also selectively evaluated: 7/23 untreated DH patients with skin symptoms, 5/23 under dapsone medication (3/5 also under intermittent GFD), 11/23 under continuous GFD. Dapsone was given to patients who wanted to get rapid improvement or received the medication in other clinics.
As a separate study 5 female pemphigus vulgaris patients and 7 healthy subjects were examined.
To examine the effect of dapsone 2 untreated DH patients and 2 healthy subjects were enrolled.

Turbidimetric clot-lysis assay and the in vitro effect of dapsone

This assay was described earlier. Briefly: Plasma clots were prepared with thrombin added to a mixture of citrated human blood plasma and tPA (Actilyse, Boehringer Ingelheim, Germany) buffer containing Ca. When serum clot lysis was examined, fibrinogen (2 mg/ml, human, plasminogen-depleted, Calbiochem, LaJolla, CA) was also added. Freshly, simultaneously prepared plasma and serum samples were analyzed within 1h after collection without freezing to avoid cryoprecipitation. The course of clot formation and dissolution was monitored by measuring the light absorbance at 340 nm (A_{340}) at 37 ^{o}C with a Zenyth 200rt microplate spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). The lysis time, defined as the time needed to reduce the turbidity of the clot to half-maximal value, was used as a quantitative parameter of the fibrinolytic activity, whereas the maximal turbidity (A_{340max}) was an indicator of the fiber size of fibrin. Higher turbidity indicates thicker fiber diameters and larger clot pores. We compared the lysis time and maximal turbidity of plasma (fibrinogen rich samples) and modified serum samples (fibrinogen-free samples supplemented with normal human fibrinogen) of patients and samples.
To analyze the effect of dapsone in vitro, it was applied at therapeutic concentration directly to freshly prepared plasma samples for 30 min prior the clotting in the fibrinolytic assay.

Scanning electron microscope (SEM) imaging of plasma clots

Three representative DH plasma samples (P1, P2, P3) and a healthy subject with average control turbidity were selected for SEM according to their plasma lysis-curves (A_{340max} values). P1 showed the highest A_{340max}, was a TG2-TG3 AB positive, untreated, P2 showed

the lowest A_{340max}, was a TG2-TG3 AB negative, dapsone and GFD treated and P3 with medium A_{340max} was a TG2 AB negative-TG3 AB positive, only dapsone treated DH patient. SEM evaluation of fibrin from further 2 untreated, anti-TG2 and TG3 IgA positive DH patients (P22, P23) and 2 healthy subjects were done to characterize the fibrin structure before and after in vitro dapsone addition.

The electron microscope imaging was done at the Institute of Materials and Environmental Chemistry of Hungarian Academy of Sciences with the manufacturer's software (EVO, Carl Zeiss GmbH, Oberkochen, Germany). We made digital photos with different magnifications to analyze the fibrin structure.

Morphometric analysis of fibrin structure

SEM images of the selected plasma clots according to the A_{340max} values were analyzed to determine the diameter of the fibrin fibers using self-designed scripts running under the Image Processing Toolbox v. 7.0 of Matlab 7.10.0.499 (R2010a) (The Mathworks, Natick, MA). For the diameter measurements a grid was drawn over the image with 10–15 equally-spaced horizontal lines and all fibers crossed by them were included in the analysis. The diameters of 300 fibers from each image were measured manually and 3 images from each plasma sample were evaluated.

Statistical procedures

The statistical evaluation of the lysis-assay parameters (lysis time, A_{340max}) was performed with Kolmogorov-Smirnov test (Statistical Toolbox 7.3 of Matlab). The distribution of the measured fiber diameter data was analyzed using an algorithm described previously to fit theoretical distributions to several empirical data sets. The best fitted distributions for different samples were compared using Kuiper test and Monte Carlo simulation procedures. When a statistically significant difference between two distributions was established, the numerical characteristics of the central tendency and variance were considered to be statistically significant. A p-value of less than 0.05 was considered statistically significant.

Results

Circulating TG3-IgA immune complex ELISA

We developed an ELISA that made detection of circulating TG3-IgA immune complexes possible in blood samples of DH patients.

There was no statistically significant difference between the plasma and serum level of immune complexes of the same patients. The circulating TG3-IgA immune complexes were significant higher in the plasma ($p=0.0124$) and the serum ($p=0.0145$) samples of DH patients compared to healthy subjects.

We did not find significant difference between the blocked and unblocked group of DH patients ($p=0.3137$) and healthy controls ($p=0.9984$) (Tukey test). The immune complex level of DH patients was significantly higher with ($p=0.0001$) and without ($p=0.0004$) BSA blocking compared with healthy subjects.

The differences between the untreated DH and the three control study groups (healthy control, PV and SLE group) were significant ($p<0.01$, Kruskal-Wallis test). The post hoc test revealed that the untreated patients with DH had significantly higher circulating TG3-IgA IC compared with healthy subjects, with patients with pemphigus vulgaris, and with patients with systemic lupus erythematosus ($p<0.01$). The difference between healthy group and pemphigus vulgaris or systemic lupus erythematosus was not significant ($p>0.5$) (multiple comparisons of mean ranks for all groups).

The IgA-TG3 circulating immune complex levels did not show a significant difference between the anti-TG3 antibody positive ($n=22$) and negative and/or marginally increased ($n=13$) groups of untreated patients with DH ($p=0.3747$, Mann-Whitney U test).
The gluten dependence of circulating TG3-IgA immune complexes was examined. The immune complexes of untreated DH patients decreased significantly under GFD, in remission ($p=0.0050$, Wilcoxon test). In some symptom-free patients with DH under GFD, however, these immune complexes never completely disappeared; they remained detectable in the circulation. There was no significant correlation between the anti-TG3 antibody levels and the

TG3-IgA immune complex values, neither within the untreated DH group (p=0.7222, Spearman rank correlation test), nor among patients under GFD (p=0.4775).

The analysis of fibrinolysis and the structure of fibrin

Turbidimetric clot-lysis assay and the in vitro effect of dapsone

We were looking for systemic factors, which affect the fibrinolytic potential of DH patients, therefor plasma clots were prepared, and lysis was induced by t-PA.

According to the lysis curves the plasma clot lysis time was prolonged in the groups of DH patients and their A_{340max} values were higher compared to healthy subjects. According to their lysis-curves plasma samples of P1 with the highest A_{340max}, P2 with the lowest A_{340max} and P3 with medium turbidity were selected for SEM.

When lysis was evaluated in clots from sera of the same patients, the kinetics of the curves changed, the lysis time and the turbidity of the healthy subjects somewhere exceeds the patients' values.

With statistical procedures the following results were obtained:

The plasma clot lysis time was significantly prolonged in the groups of untreated (n=7) as well as total DH patients (n=23) compared to healthy subjects (p=0.026, p=0.004).

The plasma A_{340max} values were significantly higher compared to healthy subjects (p=0.0009, p=0.001).

When lysis was evaluated in clots from sera of the same patients, supplemented with exogenous fibrinogen, no significant differences were detected neither in turbidity (A_{340max}), nor in lysis time in the group of untreated as well as total DH patients compared to healthy controls (lysis time p=0.1168, p=0.731, A_{340max} p=0.1698, p=0.4501). The lysis time was shorter, the A_{340max} was lower compared to healthy subjects. The removed fibrinogen and the fibrinogen-bound molecules can cause the results.

Dapsone treatment in DH resulted in statistically significant differences in the plasma clot lysis time ($p=0.01$) and A_{340max} ($p=0.02$) compared to the untreated patients, because the dapsone therapy shifted the parameters of the fibrinolytic profile towards the values of healthy subjects.

It is noteworthy that GFD improved the fibrinolytic parameters of DH patients, but statistical differences persisted in both lysis time ($p=0.023$) and A_{340max} ($p=0.01$) compared to healthy subjects.

The differences in the lysis of plasma clots from DH patients with and without dapsone therapy disappeared in serum studies when exogenous fibrinogen was added to serum samples.

A moderate (by 5–10%), but statistically significant decrease in both A_{340max} and lysis time was detected when dapsone at therapeutic concentration was applied directly to plasma samples for 30min prior the clotting in the fibrinolytic assay ($p<0.05$).

In another set of control experiments plasma clots from pemphigus vulgaris patients (n=5) showed no significant difference to plasma clots from healthy subjects (n=7), neither in A_{340max} value, nor in lysis time, and these values were also normal in their fibrinogen-supplemented serum clots.

Scanning electron microscope (SEM) imaging of plasma clots

The altered A_{340max} values observed in plasma clots from DH patients could be attributed either to variations in the fibrinogen concentration or to a modified fibrin structure. Because the fibrinogen levels of the examined plasma samples were within the normal range (1.5–4.5 g/l), we performed direct evaluation of the fibrin structure with SEM. According to their lysis-curves plasma samples of 3 patients (P1-3) and a healthy subject were selected. P1 with the highest A_{340max}, P2 with the lowest A_{340max}, P3 with medium turbidity and a healthy subject with average A_{340max} were selected for SEM.

The differences are already visible on the SEM images. The fibrin network from the untreated, TG2-TG3 AB positive P1 patient with the highest maximal turbidity showed more

convoluted and thicker fibers than the clot from the healthy subject. The fibrin in plasma clots of the TG2-TG3 AB negative, dapsone treated P2 patient on GFD presented with thinner fiber diameters and loose larger pores than P1 patient or the healthy subject.

This visual impression for existing differences in fibrin structure was further substantiated by quantitative analysis of the fiber diameter in plasma clots. The median fiber diameter in the plasma clots of the untreated P1 was larger, as compared to the fibrin of a healthy subject ($p<0.001$). The fibrin fibers in plasma clots from dapsone-treated patients, either under GFD (P2), or without diet (P3) were either thinner (P2) or identical in size (P3) compared to the control.

In a separate study SEM evaluation of fibrin from further 2 untreated DH patients and 2 healthy subjects confirmed the trend of fiber thickening in DH.

Direct application of dapsone at 5 mg/ml for 30 min prior clotting resulted in a significant reduction in fiber diameter in both DH patients and healthy subjects ($p<0.05$).

Conclusions

1. We developed a sandwich ELISA that made detection and semi-quantification of TG3-IgA immune complexes possible in DH, that support our hypothesis that DH is an immune complex disease, where circulating and precipitated TG3-IgA immune complexes in the papillary dermis are responsible for the development of skin symptoms.

2. We verified that there is no significant correlation between the anti-TG3 antibody levels and the TG3-IgA immune complex values in the group of untreated DH patients and under GFD, they can be different in the same patient. The level of the TG3-IgA immune complexes is affected by gluten and decrease significantly in remission, under GFD, the level of immune complexes changes gluten dependent. They can be present in the circulation in the absence of TG3 antibodies.

3. The t-PA induced fibrinolysis was significantly suppressed in DH patients compared to healthy subjects, so we confirmed a decreased fibrinolytic potential, an inhibited systemic fibrinolysis.

4. The impaired fibrinolytic potential at least in part can be related to the morphological alterations of fibrin structure proved by scanning electron microscopy.

5. We could confirm that dapsone improved slightly, but significantly the modified clot structure and fibrinolytic profile in vitro, and we proposed that this could be part of its therapeutic potential.

List of publications

List of publications releated to the thesis

Görög A, Németh K, Kolev K, Zone JJ, Mayer B, Silló P, Bognár P, Kárpáti S. (2016) Circulating Transglutaminase 3-Immunoglobulin A Immune Complexes in Dermatitis Herpetiformis. J Invest Dermatol. 136: 1729-1731.

IF:6,915

Görög A, Németh K, Szabó L, Mayer B, Silló P, Kolev K, Kárpáti S. (2016) Decreased fibrinolytic potential and morphological changes of fibrin structure in dermatitis herpetiformis. J Dermatol Sci. 84: 17-23.

IF:3,739

List of publications not releated to the thesis

Tukaj S, **Görög A**, Kleszczyński K, Zillikens D, Kárpáti S, Kasperkiewicz M. (2016) Autoimmunity to heat shock proteins and vitamin D status in patients with celiac disease without associated dermatitis herpetiformis. J Steroid Biochem Mol Biol. doi: 10.1016/j.jsbmb.2016.10.002. [Epub ahead of print]

IF:3,985

Kasperkiewicz M, Tukaj S, Gembicki AJ, Silló P, **Görög A**, Zillikens D, Kárpáti S. (2014) Evidence for a role of autoantibodies to heat shock protein 60, 70, and 90 in patients with dermatitis herpetiformis. Cell Stress Chaperones, 19: 837-843.

IF:3,163

Orbán Annamária, **Görög Anna**, Silló Pálma, Kuroli Enikő, Hársing Judit, Kárpáti Sarolta. (2014) Gyermekkori lichen sclerosus et atrophicus, alopecia totalis és autoimmun thyreoiditis együttes előfordulása. Bőrgyógyászati és Venerológiai Szemle. 90: 55-59.