The role of transglutaminase 3 in dermatological disorders

Ph.D. Thesis

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INTRODUCTION

My thesis describes the so far unrecognized role of transglutaminase 3 (TG3) in skin pathology, and reviews its functions related to dermatology. The thesis is built around two major themes, divided into two major sections accordingly.

Firstly I describe the latent skin barrier defect detected in transglutaminase 3 knockout (TGM3 -/-) mouse strain, based upon our epicutaneous sensitization studies and *in vivo* dye penetration assays.

In the second part I discuss the unusually high prevalence of cryofibrinogenemia found in dermatitis herpetiformis (DH) patients treated at Semmelweis University, Department of Dermatology, Venereology and Dermatooncology. TG3 comes up as an autoantigen in dermatitis herpetiformis.

Latent skin barrier defect in TGM3 -/- mice

Latent skin barrier defects were identified primarily as an important background of atopic diseases and contact dermatitis.

The cornified cell envelope (CE) a complex protein-lipid structure - being the outermost skin barrier is in direct contact with the environment, thus it is of great significance in skin barrier function.

The members of the transglutaminase (TG) enzyme family – TG1, TG3 and TG5 – are prioritised in formation of CE by producing stable isopeptide bonds between skin structure proteins.

The insufficient transglutaminase activity in keratinization results in a modified CE structure, leading to skin barrier defect. The recently published TGM3 -/- mouse strain showed no other barrier dysfunction, but late *in utero* epidermal development, abnormal hair structure and increased isolated corneocyte fragility.

Nevertheless, based on the significant role of transglutaminases in CE formation, we presumed latent skin barrier defect, predisposing to enhanced percutaneous sensitization. We examined the animals in the well-documented fluorescein-isothiocyanate/dibutyl-phtalate (FITC-DBP) contact dermatitis model.

Cryofibrinogenemia in dermatitis herpetiformis

Dermatitis herpetiformis is one of the gluten-sensitive entheropathy (GSE, celiac disease) -associated diseases. In GSE, transglutaminase 2 (TG2) is the autoantigen. However in DH, the similar-structured TG3 becomes the main autoantigen through epitope spreading.
We started to examine the presence of cryoglobulin and cryofibrinogen in dermatitis herpetiformis populations based on the intermittently occurring acral purpuras in DH.

AIMS/OBJECTIVES

1. The recently produced TGM3 -/- mouse strain showed no manifest skin barrier defect. Based upon the role of transglutaminases in CE formation, we presumed latent skin barrier defect in the knockout mice. According to this, we aimed to analyse the functionality of the cutaneous barrier with antigen load in the FITC-DBP contact dermatitis model. Assuming that the TGM3 -/- mouse strain shows no difference in immune reactivity compared to the wild-type (WT) mice, the increased inflammatory reaction to equal epicutaneous antigen stimulation proves the latent skin barrier defect.

2. Besides the investigation of the TGM3 -/- mouse FITC-DBP contact dermatitis model, we wanted to examine the immune response of TG3 deficient mice through bypassing the cutaneous barrier.

3. Furthermore we planned to develop a new *in vivo* method – two-photon absorption fluorescence microscopy – to follow the in vivo percutaneous FITC penetration.

4. Considering the general decline of skin barrier function in the elderly, we aimed to investigate and compare the sensitization of TGM3 -/- and WT mice with different age groups (8-12 weeks, 6 months, 18 months) in FITC-DBP model.

5. TG3 is the pathognomonic autoantigen of DH. We started to analyse the presence of cryoglobulin and cryofibrinogen in DH patients based on the intermittently occurring acral purpuras in DH. The analysed population consisted of 88 patients with DH who had been treated at our department. We aimed to investigate the prevalence of cryoproteins in the mentioned population and to analyse the potential effect of gluten-free diet and dapsone treatment.

METHODS

TGM3 -/- mouse

TGM3 -/- mouse strain was produced in the University of Cologne with neomycin resistance gene technology in TGM3 allele exon 6 of C57BL/6 WT strain. We used the animals in collaboration with the University of Cologne and tested with ethical permission of Animal Testing Ethical Council, Semmelweis University (22.1/1049/3/2010).

The male and female knockout (KO) animals were both viable and able to produce homozygote offspring. The animals were kept in 12-hour light-dark cycles, apart from the time of the testing with free food and fluid access.
Sensitization tests, mouse ear swelling test (MEST) and histological evaluation were all performed in three age groups: 8-12 weeks, 6 and 18 months. The flow cytometric, serum IgE measurements, the P. acnes in vivo assay and the two-photon microscopy measurements were performed only in 8-12 week old mice.

Contact sensitization provocation – mouse ear swelling test (MEST)

We used the FITC-DBP model to provoke delayed hypersensitivity reaction. Around 2x2 cm area of the mice's abdomen was shaved off (day 0) and after 24 hours (day 1) in a standard aluminium chamber 0.5% FITC dissolved in 160µl acetone/DBP 1:1 v/v mixture was placed on the shaved area, and kept for 24 hours. (For the control group, the same amount of solvent was used). On the 7th day of the experiment, the exposed area was shaved again, then on next day (Day 8), the 24-hour exposure was repeated. A week later (day 15) we measured the basal ear thickness (0h) with digital micrometer. Then we pipetted on both sides of the dorsal ear surface 20µl of 0.5% FITC and acetone/DBP 1:1 (v/v) solution in the control group. Ear thickness was measured again in 24 and 48 hours. The average rate measured in both ears was used for statistical evaluation. The ear swelling rate was proportional with the inflammatory reaction.

Histology

After re-exposition at 48 hours, ears were removed, fixed in 10% buffered formalin, then prepared with standard dehydration and

embedding protocols. The 2 μm cuts were analysed after hematoxylin-eosin (HE) and toluidine blue staining.
We evaluated the epidermal thickening, crust formation in the epidermis and the inflammatory infiltrate in the dermis. In the toluidine blue stained cuts, we examined the average number of mast cells (subepidermal and metachromatic dying) in 5 viewfields, with 200x enlargement. The inflammation and the mast cell number was evaluated using a semi-quantitative scale (0,+,++).

Flow cytometric measurements

The prepared lymph nodes were homogenised in sterile PBS. For the cell phenotype analysis we used the following conjugated antibodies: PE-conjugated anti-mouse CD3, PerCP-conjugated anti-mouse CD4, and PE-conjugated anti-mouse CD25 (BD Biosciences, San Jose, CA).
We used the bench-top flow cytometer system (FACSCalibur) for measurement, evaluated the results with the CellQuest Pro programme. We used 5-5 8-12 week old animals. The threshold was set to 10^4 in all samples. Cell populations were identified on forward scatter (FSC) – side scatter (SSC) and dot-plot graphs.
The CD4, CD25 double positive cells were equal to activated T-cells; we examined its rate within the lymphocyte gate. We generated histograms on FITC emission wavelength (518-520 nm), as the hapten used for sensitization was FITC.

Transepidermal FITC penetration *in vivo* measurement with two-photon absorption fluorescence microscopy

During the in vivo measurements we anesthetized the animals, affixed their ears to the slide and pipetted 2 μl FITC dissolved in 50 μg/ml DMSO (dimethyl sulfoxide) on the dorsal side of the ear. After 15

minutes, we followed the FITC penetration with two-photon microscope. We used Ti-sapphire laser for sample induction (76 MHz frequency, 190 femtosecond impulse). The fluorescent sign was well detectable and the tissue damage could be avoided.
We used Carl Zeiss microscope (LSM 7MP) with 20x immersion objective for detection. Penetration was registered after 15, 30, 40 minutes. We performed optical slicing on an 850 μm x 850 μm field, from stratum corneum to the dermis in 80 μm depth.
The 3D graphics and the fluorescence intensity data were processed with ZEN software (Carl Zeiss); the picture analysis was performed with the UTHSCA Image Tool for Windows 3.0 programme. We used the 30 min z-stack records of three 8-12 weeks old TGM3-/- and WT animals per group.

In vivo Propionibacterium acnes assay

Subepidermally injected P. acnes provokes massive inflammatory reaction in mourine ears. We injected 20μl 10^{14} CFU P. acnes strain dissolved in sterile PBS in the left ear of the mice; the right ear was used as control, which was treated with 20μl sterile PBS. The degree of inflammation was evaluated after 48 hours by MEST based on the difference in thickness between the right and the left ear. We used three 8-12 weeks old TGM3-/- and WT animals per group.

Serum IgE ELISA

At the 48 hour evaluation of ear thickness, sera were obtained from both FITC-treated TGM3/KO and WT mice, and kept frozen until ELISA measurements. Total serum IgE levels were measured with a commercially available mouse IgE ELISA KIT (Mouse IgE ELISA set, BD Biosciences, San Jose CA) together with the additional reagent set (BD Biosciences, OptEIA Reagent Set B) according to instructions

provided by the manufacturer. We used five 8-12 weeks old TGM3-/- and WT animals per group.

Cryofibrinogenemia in dermatitis herpetiformis

We analysed the presence of cryofibrinogen and cryoglobulin in patients with direct immunofluorescence and histologically proved DH – independently of disease activity and serology markers. We processed the data of 88 DH patients: 60 men and 28 women, the average age was 36.5±17.4 years. We retrospectively processed the data of cryofibrinogen (CF) and cryoglobulin (CG) examinations carried out in the laboratory of Semmelweis University, Department of Dermatology, Venereology and Dermatooncology in a period of 2 years. The subjects of these examinations were patients diagnosed or examined for autoimmunity (SLE, dermatomyositis, Raynaud-phenomenon, different types of vasculitis), and a smaller rate of other dermatologic conditions (ulcus, urticaria, livedo reticularis, mycosis fungoides). In this period, 233 examinations for CF and CG were performed in non-DH patients. This group consisted of 56 men and 177 women; the average age was 52.9±17.4 years.

We investigated the prevalence of cryofibrinogenaemia in the following DH subpopulations: on gluten-free diet or treated with dapsone and gluten-free diet.

Detection of Cryofibrinogen (CF) and cryoglobulin (CG)

Cryofibrinogen determination: blood samples were taken from the patients to Vacutainer® native tubes previously warmed up to 37 ºC. 1 ml sterile 2.8% sodium-citrate solution was added to the blood to inhibit coagulation. The sample was centrifuge-cooled to 4ºC for 20 minutes

on 2000 RPM speed. Plasma kept on 4ºC was evaluated quantitatively for precipitate (cryofibriogen) after 72 hours. The control was a tube of plasma kept on 37 ºC.

Cryoglobulin determination: blood samples were taken from the patients to Vacutainer® native tubes (37ºC), then incubated on 37 ºC in tubes for 3 hours. Then the sera was centrifuged (2000 RPM, 20 min), kept on 4ºC was evaluated quantitatively for precipitate (cryoglobulin) after 72 hours. The control was a tube of sera kept on 37 ºC.

Statistical analysis

We used parametric Student-type T probe (Mann-Whitney test). P probability value was considered significant if lower than 5%. We used IBM SPSS Statistics 19 Software-t (IBM, Armonk, NY) for analysis.

RESULTS

MEST results in 8-12 weeks, 6 and 18 months TGM3 -/- and WT mouse populations

8-12 weeks population: The average increase in ear thickness (ear thickening, ΔET) among the FITC-treated TGM3 -/- mice (n=20) was 18±13 μm at 24hours, and 61±19μm at 48hours. In the group of FITC-treated WT animals (n=9), ΔET was 6±3μm at 24hours and 7±9μm at 48hours. The 48h MEST value proved to be significantly higher ($p \leq 0.0001$) in the FITC-treated TGM3 -/- group. In the vehicle-treated TGM3 -/- group (n=12), the average extent of ΔET was 3±4μm at 24 hours and 17±14μm at 48 hours, whereas in the vehicle-treated WT group (n=9) this value was 6±3μm at 24 hours, and 7±9μm at 48 hours, respectively, showing no significant difference.

6 months population: The ΔET among the FITC-treated TGM3 -/- mice (n=8) was 41±23μm at 24hours, and 70±28μm at 48hours. In the group of WT animals ΔET was 18±7μm at 24hours and 24±9μm at 48hours. The 48h ΔET in this age group was significantly higher again ($p \leq 0.0001$) among FITC-treated TGM3 -/- mice. Upon vehicle treatment, in TGM3 -/- group (n=6) ΔET was 3±12μm at 24 hours and 7±4μm at 48 hours, while in the WT group (n=5) the values were 3±3μm and 5±7μm respectively. Thus, with vehicle-treatment in every group we observed negligible ΔET.

18 months population: The ΔET value among the FITC-treated TGM3 -/- mice (n=5) was 30±16μm at 24hours and 52±18μm at 48hours. In the group of WT mice (n=5) this value was 16±11μm at 24hours and 25±11μm at 48hours. Again, the ΔET measured in 48 hours were significantly higher among FITC-treated TGM3 -/- mice in this age group as well compared to the age-matched WT group ($p \leq 0.001$).
In the vehicle-treated TGM3 -/- group (n=5), the MEST was 3±3μm at 24 hours and 7±6μm at 48 hours, whereas in the vehicle-treated WT group (n=5) this value was 3±3μm at 24 hours and 5±5μm at 48 hours respectively, showing no significant difference.
According to the MEST data, the eldest animals could be sensitized as well, but we could not verify significant difference in ΔET between different age groups in either 24 or 48 hours.

Histology results (all age groups)

The histology results correlated well with the MEST results in all age groups. In histological analysis, we found similar differences in all populations. There were no inflammatory signs with vehicle-treatment in either TGM3 -/- or WT mice.

As a result of FITC treatment in WT group, moderate inflammatory cell dermal infiltrate and slight epidermal thickening could be detected. In the FITC-treated TGM3 -/- group, there was a higher rate of dermal inflammatory infiltrate and epidermal hyperplasia, also we could find crusting which was very rare in WT group.
The average mast cell number was more than 10/viewfield in both FITC and vehicle-treated groups on toluidine blue stained cuts and there was no significant difference according to age.

Flow cytometry

The activated T-cell proportion in draining lymph nodes was significantly ($p \leq 0.01$) higher (24.2±2.5%) in FITC-treated TGM3 -/- mice, than in FITC-treated WT group (8.2±3.3%). In the vehicle-treated populations of knockout and wild type mice there was no significant detectable difference in the number of activated T-cells (WT group: 1.4±0.8%; TGM3 knockout group 2.5±1.1%). The fluorescence detected on FITC wavelength in the non-lymphoid gate, in homogenized lymph nodes (not treated with antibody) tended to a higher rate in KO animals, but the difference between KO and WT group was negligible.

Serum IgE level

The total IgE level was 868±107 ng/ml in FITC-treated WT animals, while it was significantly higher in the TGM knockout group: 2810±796 ng/ml ($p \leq 0.05$). In the naïve, untreated WT mice the total IgE level was measured as 72±35 ng/ml, while in TGM knockout animals the value was 87±27ng/ml.

In vivo Propionibacterium acnes assay

The TGM3 -/- strain showed 27±19.3% ET, while the WT strain had 25±12.2% ET compared to the opposite ears treated with sterile PBS. The difference was not significant statistically (p=0.2254, Mann-Whitney test).

Transepidermal FITC penetration measurement with two absorption fluorescence microscopy

FITC could penetrate fast in TGM3 -/- mice epidermis and after 30 minutes in a depth of 20 μm we could detect a visible fluorescence "frontline". In the WT mice FITC spent more time in the surface of epidermis, the fluorescent sign was pale with blurred pattern. After evaluation of the records, the relative total fluorescence intensity was 4.5 ± 0.5x higher in TGM -/- mice compared to WT animals.

Cryofibrinogenaemia prevalence in DH patients

Among unselected DH patients (n=88), the prevalence of isolated CFE was unexpectedly high, reaching 48.9% (43/88), compared to 27.5% (64/233) measured in the controls.

Isolated cryofibrinogenemia (CFE) was detectable in a very high rate, in 33/55 (60%) patients under normal diet without dapsone, whereas in patients already on gluten-free diet (GFD) but no dapsone, CFE was present in a lower rate: 10/25 (40%). In the group of patients on GFD and concomitant DPS treatment CFE was not detectable in the eight studied patients (0/8).

We did not observe similar changes of cryoglobulinemia and mixed cryofibrinogenia–cryoglobulinemia in subpopulations with DH.

CONCLUSIONS

1. Investigating the deficiency of TG3 – a component of stratum granulosum – in TGM3 -/- mice, we concluded, that the knockout animals show significantly increased sensitizatory predisposition, thus decreased percutaneous inflammatory threshold compared to C57BL/6 WT mice in FITC-DBP model. We consider this result in TGM3 -/- mice as an indirect sign of skin barrier defect.

2. TGM3 -/- and WT mice reacted similarly to Propionibacterium acnes antigen stimulus upon bypassing the epidermis allocated directly into the dermis. In consequence, the increased percutaneous sensitization observed in TGM3 -/- mice is not a result of a TG3 deficiency-related different immunologic reactivity, but an increased percutaneous antigen penetration – caused by abnormal skin barrier.

3. We examined the cutaneous barrier with a new method – two-photon absorption fluorescence microscopy. We could verify the increased (and different pattern) penetration of FITC, as fluorophore through the TGM3 -/- mice's skin. The newly developed procedure is therefore suitable to examine latent skin barrier defects.

4. All ages of TGM3 -/- mice (8-12 weeks, 6 and 18 months) showed increased sensitization compared to the same aged WT mice in FITC-DBP model. At the same time, the degree of sensitization was independent of the age and hasn't changed significantly in either TGM3 -/- or WT mice. Considering this, we can hypothesize that FITC-DBP penetration doesn't change significantly in correlation with age.

5. Cryofibrinogenaemia appeared at a high rate (48.9%) in the group of unselected dermatitis herpetiformis patients (n=88). The prevalence of

cryofibrinogenaemia was even higher in patients without gluten-free diet. It follows that the prevalence was decreased in patients on gluten-free diet and it was the lowest in patients treated with dapsone and gluten-free diet simultaneously.
The high prevalence of cryofibrinogenaemia detected in dermatitis herpetiformis may be the reason for the frequent occurrence of acral purpuras in the disease.

SUMMARY

My thesis describes the previously unrecognized role of transglutaminase 3 in skin pathology and reviews its functions in dermatology.
The main role of transglutaminases is to produce resistant supramolecular structures with forming stable isopeptide bonds. These structures provide a physico-chemical barrier. TG1, TG3 and TG5 are important in the formation of stratum corneum and the CE in the skin.
On the other hand, the disturbance of barrier function frequently leads to eczematic diseases (e.g. atopy, contact dermatitis), caused by the significantly increased susceptibility to contact sensitization.
The role of other elements of the CE is an intensively researched area; still the similar function of transglutaminase 3 – localized in the same region – so far has not been investigated.
According to this, we found the intact skin barrier function of TGM3 -/- mice in described standstill conditions unexpected. We hypothesized that the TGM3 -/- mouse skin barrier dysfunction is present in a latent manner, so as a result of stress conditions – i.e. antigen exposure – it behaves in a different way compared to control mice skin and may show increased susceptibility for sensitization. Therefore TGM 3 -/- mice were examined in an FITC-DBP experimental contact dermatitis

model, which could justify the increased sensitization of knockout mice in ages of 8-12 weeks, 6 and 18 months as well. After it was confirmed that the immunoreactivity of TGM3 -/- mice does not differ from that of the WT type, based on the observed increased sensitization, we concluded that the skin barrier function was damaged in the TGM3 -/- mice and latent barrier dysfunction could be demonstrated.

Furthermore we developed an *in vivo* method using two-photon microscopy to investigate the skin barrier which allowed us to demonstrate the skin barrier defect of TGM3 -/- mice directly.

In the second part of my thesis I analysed the celiac disease-associated DH, as in this disorder the TG3 acts as an autoantigen and shows its only so far verified pathognomonic role. I started to analyze the unusually high prevalence of cryofibrinogenaemia found in DH patients treated in our department.

The prevalence of cryofibrinogenemia was lower in the group on gluten-free diet, whereas the prevalence was even lower in the group treated with dapsone and gluten-free diet.

The high prevalence of cryofibrinogenaemia detected in dermatitis herpetiformis can be an explanation for the frequent occurance of acral purpuras, in addition, it proposes a possible new mechanism of action for dapsone.

In the background of dermatitis herpetiformis, atopic diseases and celiac disease have a common feature: a barrier (skin, mucosa or bowel) exposed to the noxas of the environment is the primary location of pathological events. The role of the primer barrier dysfunction – beside the immunologic predisposition – is a feasible factor in the two previously mentioned diseases, in which transglutaminases are a considerable contributory factor.

PERSONAL PUBLICATIONS
Thesis-related publications

1. Bognar P, Nemeth I, Mayer B, Haluszka D, Wikonkal N, Ostorhazi E, John S, Paulsson M, Smyth N, Pasztoi M, Buzas EI, Szipocs R, Kolonics A, Temesvari E, Karpati S. (2014) Reduced inflammatory threshold indicates skin barrier defect in transglutaminase 3 knockout mice. J Invest Dermatol, 134: 105-111. **IF: 7.216**

2. Bognár P, Görög A, Kárpáti S. (2014) High prevalence of cryofibrinogenaemia in dermatitis herpetiformis. J Eur Acad Dermatol Venereol, 2014 Dec 10. **IF: 3.029**

3. Bognár P, Temesvári E, Németh I, Hársing J, Kuzmanovszki D, Kárpáti S. (2015) Fluoreszcein-izotiocianát kiváltotta fokozott perkután szenzibilizáció, különböző életkorú transzglutamináz-3 knockout egerekben. Bőrgyógy Venerol Sz, 91: 5-9.

Other publications

1. Bánvölgyi A, Balla E, Bognár P, Tóth B, Ostorházi E, Bánhegyi D, Kárpáti S, Marschalkó M. (2015) Lymphogranuloma venereum: the first Hungarian cases. Orv Hetil. 156: 36-40. **IF:0.291**

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3. Fodor K, Bognár P, Kiss J, Holló P, Marschalkó M, Szlávik J, Bánhegyi D, Kárpáti S. (2011) Bőrtünetek alapján diagnosztizált HIV esetek Bőrgyógy Venerol Sz, 87: 149-154.