Thesis for the degree of Doctor of Philosophy (Ph.D.)

ROLE OF TISSUE TRANSGLUTAMINASE IN PHAGOCYTOSIS OF APOPTOTIC CELLS BY MACROPHAGES

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INTRODUCTION

Phagocytosis

Programmed cell death generates a large number of apoptotic bodies that must be cleared to avoid secondary necrosis, tissue damage and inflammatory response. Apoptotic cells are usually cleared by phagocytosis *in vivo* and this process is mediated by either professional phagocytes, called macrophages or by amateur ones, which are usually the neighbours of dying cells. Phagocytic receptors recognize specific 'eat-me' signals on the apoptotic cell, through either direct apoptotic cell–phagocyte interactions or serum opsonizing proteins that bridge apoptotic ligands and the phagocyte receptors. The redistribution of phosphatidylserine (PS) on the surface of the apoptotic cell is the best characterized mark that distinguishes cellular life from death. Several phagocytic receptors are attracted to PS through direct or indirect interactions mediated by bridging molecules such as milk fat globule EGF-factor 8 (MFG-E8), which is secreted by activated macrophages. Phagocytic receptors, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins bind an RGD motif in the EGF-like domain of the soluble molecule MFG-E8. Besides PS-binding bridge molecules T-cell immunoglobulin- and mucin-domain-containing molecule (Tim4), brain-specific angiogenesis inhibitor 1 (BAI1) and stabilin-2 multifunctional receptors were identified as novel PS-binding phagocytic receptors for apoptotic cells.

Low-density lipoprotein receptor–related protein 1 (LRP1), is another recognition receptor on the macrophage surface. LRP1 interacts

with calreticulin (CRT), which is highly expressed on the surface of apoptotic cells. LRP1 transduces the signal via a phosphotyrosine binding motif within their cytoplasmic tails to the adaptor protein, GULP (engulfment adaptor protein).

It is generally believed that different surface receptors on the phagocyte converge on two evolutionally conserved pathways upstream to the activation of the low molecular weight GTPase Rac1, which is obligatorily required for the uptake. The first pathway is initiated by either LRP-1 or stabilin-2 as receptors and the adaptor protein GULP, and regulates Rac1 activity by yet unknown mechanisms. The second pathway is mediated via the 180 kDa protein downstream of chicken tumor virus no. 10 (CT10) regulator kinase II (Dock180) and the engulfment and migration protein (ELMO), which form together an unconventional two-part guanine nucleotide exchange factor for Rac1. αvβ5 integrin-dependent phagocytosis activates a signal cascade involving CrkII/ DOCK 180/ Rac1 to promote early phagosome formation. Another upstream pathway of Rac involves RhoG and the guanine nucleotide exchange factor TRIO. αvβ3 integrin induced apoptotic cell engulfment, in the presence of MFG-E8, could be inhibited by dominant-negative forms of RhoG or Rac1, which suggested that RhoG and Rac1 are also involved in the integrin-mediated engulfment.

Tissue transglutaminase (TG2)

Transglutaminases are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond

between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in target proteins. Upon activation by Ca^{2+}, TG2 contributes to the organization of cytoskeleton by crosslinking various cytoskeletal proteins, i.e. microtubule protein, tubulin, actin, myosin. Extensive polymerization, which occurs during the final steps of apoptosis, stabilizes the structure of the dying cells thereby preventing release of cell components that might give rise to inflammatory or autoimmune responses.

In signaling TG2 can act as a G protein. G protein function of TG2 has role in modulating dynamic adhesion formation in cell spreading and migration, taking part of intracellular signal transduction resulting activation of protein kinase Cα. The GDP/GTP-bound form cannot act as a transglutaminase. This inhibition is suspended by Ca^{2+}, which serves as a switch between the two distinct functions. Additionally, TG2 can act as an adaptor protein that facilitates extracellular interaction between fibronectin and integrins**.** Fibronectin binding is believed to play a role in localizing TG in regions of tissue damage, where it acts as a matrix-associated membrane-bound exoenzyme.

Structure of TG2

TG2 has four distinct domains: an N-terminal β-sandwich (with fibronectin and integrin binding site), catalytic core (containing the catalytic triad for the acyl-transfer reaction: Cys277, His335, and

Asp358) and transition state stabilizing residue (Trp241) and two C-terminal β-barrel domains. It was shown that Tyr274 is important for the externalization mechanism of the enzyme. Mutation of Tyr274, thought to provide *cis* rather than the preferred *trans* peptide bond conformation, leads to lack of enzyme secretion. The GDP-binding site of TG2 is located in a hydrophobic pocket between the core and β-barrel 1, on the opposite face to the proposed glutamyl substrate-binding site. TG2 can interact with fibronectin with its N-terminal region. The β5/β6 hairpin of the first domain represents the major recognition site on the TG2 molecule for the interaction with fibronectin. TG2 can also bind to integrin receptor on the cell surface, but the exact location of this binding site has not indentified yet. Importantly, the interaction between TG2 and integrin is independent of the cross-linking activity of TG2.

Role of TG2 in apoptosis and phagocytosis

In apoptosis. TG2 has both pro-and antiapoptotic effects depending upon the type of cell, kind of death stimuli, the cellular localization of the enzyme and the type of activities switched on.

Tissue transglutaminase is induced and activated in cells undergoing apoptosis in the liver and thymus forming highly cross-linked protein polymers and proteinaceous shells which were resistant to detergents. TG2 with its BH3 domain can induce conformational change and translocation of Bax to the mitochondria, release of cytochrome *c* and death of neuroblastoma cells. Following the initiation of apoptosis the enzyme also could transamidate the actin and

retinoblastoma (Rb) proteins but in the other hand transamidation of Rb TG2 can protect Rb from caspase-induced degradation.

Externalized TG2 by binding to fibronectin and cell surface heparan sulfate chains supports the adhesion-dependent survival signaling via activation of Rho and the focal adhesion kinase.

TG2 also participates in clearance of apoptotic cells. In one hand TG2 could promote phagocytosis from the side of apoptotic cells by facilitating the phosphatidylserine exposure or by crosslinking the S19 ribonuclear protein that acts as chemotactic factor for macrophages but the main defect was found in macrophages. This was partially related to a defect in TGFβ activation, which promotes phagocytosis of apoptotic cells. Main role of TG2 *in vivo* to ensure that apoptosis has been initiated, it is finished without causing inflammation and apparent tissue injury.

However, macrophages lacking TG2 exposed to recombinant TGFβ (1μg/ml) in short term culture are still much less efficient in engulfing apoptotic cells than $TG2^{+/+}$ macrophages suggesting that other mechanisms must also contribute to the observed delay in phagocytosis of $TG2^{-/-}$ cells.

AIMS

Previous studies in our laboratory have revealed the loss of TG2 in mice leads to an *in vivo* defect of phagocytosis of apoptotic cells, and the defect affects the macrophage site. The main aim of the present study was to investigate whether the defect also exists *in vitro* and to characterize the deficiency that develops in the engulfment process in the absence of TG2.

For this purpose we decided

1. To set up a phagocytosis model and techniques with the help of which the phagocytosis of apoptotic cells by macrophages can be studied *in vitro*.
2. To characterize the phagocytosis of apoptotic cells by wild-type macrophages using time-lapse video.
3. To characterize the phagocytosis of apoptotic cells by $TG2^{-/-}$ macrophages using time-lapse video.
4. To develop an adenoviral gene delivery system to determine which biological function of the TG2 is required for the phagocytosis process.
5. To characterize changes in the phagocytic receptors expression which try to compensate the loss of TG2.
6. To characterize the changes in the phagocytic signaling pathways that develop in the absence of TG2.

METHODS

Cells

Peritoneal macrophages derived from $TG2^{+/+}$ and knock out mice were used for phagocytosis experiments. Mice were injected with 2ml 4% thioglocolate and four days after peritoneal lavages were performed to receive differetiated macrophages. 5x 10^5 peritoneal macrophages were allowed to adhere in 24-well plates for 24 hours in the present of 10μM red fluorescent CMTMR at 37°C/ 5%CO_2. Thymocytes from 4 weeks-old WT mice were used as apoptotic target cells. Isolated thymocytes were labelled overnight with 6 μM green fluorescent CFDA, and subsequently treated with 4 μM ionomycin for 6 hrs. 40–50% of thymocytes were Annexin V positive (*i.e.* apoptotic), and less than 5% of Annexin V positive cells were propidium iodide positive (*i.e.* necrotic). Thymocytes and macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, L- glutamine, penicillin and streptomycin.

Phagocytosis assay

Macrophages were incubated with 2-μm carboxylate-modified red fluorescent beads or with apoptotic thymocytes labeled with CFDA in 40:1 target/macrophage ratio. Cells incubated with apoptotic thymocytes at 4 °C were used as controls. For competition experiments necrotic thymocytes were killed pathologically by incubation at 55°C for 10-15 min. After washing, the cells on the plate were trypsinized,

resuspended in cold medium with 0.5% sodium azide, and 10,000–20,000 cells were analyzed for each point by two-color flow cytometry.

Site-directed mutagenesis

Site-directed mutagenesis was performed on plasmid pSP73 carrying the murine TG2 gene. The Quik-Change Site-Directed Mutagenesis Kit was used for introduction of the desired mutations. The following primers were used for cross-linking deficient mutant: TG2-X up: 5'-GTGAAGTACGGGCAGAGTTGGGTGTTTG-3' and TG2-X down: 5'-CAAACACCCAACTCTGCCCGTACTTCAC-3'; for guanine nucleotide-binding site mutants: TG2-G1 up: 5'-CTACCAAGGCTCTGTCAACGACATCAAGAGTGTGCC-3' and TG2-G1 down: 5'-GGCACACTCTTGATGTCGTTGACAGAGCCTTGGTAG-3;' TG2-G2 up: 5'-CAGCTACCTGCTGGCTCAAGAAGATCTCTACCTGGAG-3' and TG2-G2down: 5'-CTCCAGGTAGAGATCTTCTTGAGCCAGCAGGTAGCTG-3'; for fibronectin binding deficient mutant: TG2-FN up: 5'-CAGTGCTGGCCCAACAGGCCAATGTCCTCTC-3' and TG-FN down: 5'-GAGAGGACATTGGCCTGTTGGGCCAGCACTG-3'; for secretion deficient mutant: TG2-S up: 5'-CACCCAGCACTGCCCGGACTTCACTTGCTG-3' and TG2-S down: 5'-CAGCAAGTGAAGTCCGGGCAGTGCTGGGTG-3'.

Adenoviral gene delivery system

Recombinant, replication-deficient adenoviral vectors encoding either LacZ, the murine TG2 gene, the different TG2 mutants, wild type and constitutive active Rac genes were produced using the AdEasy

XL system according to the manufacturer's instruction. For gene delivery, 10^6 macrophages were exposed to $2x10^9$ plaque forming units (PFU)/ml virus particles for 48 hrs in the same medium. LacZ expression was determined with X-gal staining, while TG2 and Rac expression by Western blot analysis.

Biotinylation and isolation of cell surface proteins

Cell monolayers (corresponding to $4x10^6$ cells) were rinsed with ice-cold PBS and preincubated in PBS on ice for 45 min prior to further incubation with Biotin-XX SSE dissolved in PBS (0.5 mg/ml) for 20 min. Cells were scraped in lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM EGTA) and sonicated. Lysates were boiled for 5 min, cleared by centrifugation (13,000 rpm, 20 min) at room temperature, and incubated with NeutrAvidin-agarose. Proteins bound to NeutrAvidin-agarose were separated by 10% gel electrophoresis and analyzed by Western blot analysis by using anti-tissue transglutaminase antibodies.

Q-PCR analysis for detecting of the expression of various phagocytosis receptors

ABI PRISM 7700 Sequence Detection System was used to determine relative gene expression. 18S ribosomal RNA was used as endogenous control to normalize the amount of the sample cDNA added to the reaction. The 18S primers were labeled with VIC and sample primer with FAM. All samples were run in triplicate. Relative

mRNA expression was quantified by comparing the cycle threshold (CT) between control and knockout cell samples.

Immunofluorescence staining and confocal microscopy

Peritoneal macrophages isolated from WT and $TG2^{-/-}$ mice were plated in two-well chamber-slides ($5x10^5$/well) and cultured for 48h before used. After exposing or not macrophages to apoptotic cells for 30 min, cells were washed than fixed in ethanol/ acetone 1:1 for 10min at -20 °C. F-actin was stained with of phallotoxin-Alexa488 for 20 min at room temperature. Samples were examined with a Leica TCS SP confocal laser scanning microscopy. For integrin β_3 and LRP staining macrophages were blocked with 50% FBS for 30 min at 37˚C, then washed with ice cold HEPES buffer and stained with phycoerytrin labelled hamster anti-β_3 integrin or anti-LRP antibody for 15 min on ice. For LRP staining, Alexa 647 anti-goat IgG was used as a secondary antibody. After washing with HEPES buffer cells were fixed with 4% paraformaldehyde for 20 min. For detecting Rac1 cells were labelled with purified mouse anti-Rac1 monoclonal antibody for 30 min at room temperature. After washing Alexa 647-conjugated goat, anti-mouse IgG was used as secondary antibody. Images were taken with a Zeiss LSM 410 or Olympus FV1000 confocal laser scanning microscope. For visualizing the distribution of integrin β3 and Rac1, overview images and 3D stacks were acquired at 1 μm optical thickness. 3D reconstructions and XYZ projections were created with the LSM 4.0 software.

Detection of active Rac1 and RhoG

Macrophages plated overnight were exposed to 2 μm carboxylated latex beads for 40 min, or left untreated as controls, to detect GTP-bound forms of Rac1 and RhoG. Pull down assay was performed with the EZ-Detect Rac1 Activation Kit, according to manufacturer's instruction. For the RhoG pull-down assay PAK-GST was replaced with an ELMO-GST protein. Active RhoG was detected by Western blot analysis using anti-RhoG antibody. For these assays 1-1.5 mg of total cell proteins (determined by the Bradford method) was used in the pull-downs.

Biacore assays

Surface plasmon resonance experiments were performed on a Biacore 3000 instrument equipped with research grade CM5 sensor chips. Anti-GST surfaces were prepared at 25 °C by the amine-coupling method as recommended by the manufacturer. To capture human recombinant GST-TG2 or GST, these proteins were injected over the anti-GST surface at 2.5 μM concentration. To monitor association, various concentrations of recombinant human MFG-E8 or fibronectin were applied to either the GST or GST-TG2 surface at a flow rate of 10 μl/min for 7 min and the changes in the response units (RU) were recorded. Dissociation of the complexes was followed for up to 6 min after initial binding. The responses from the surface covered with GST were subtracted from the responses obtained with the GST-TG covered surfaces. Kinetic (k_a and k_d) and equilibrium parameters (K_a) were derived from the sensograms using

BIAevaluation 3.1 software with fitting the data to a simple 1:1 Langmuir interaction model.

Binding of TG2 to lipids

Membrane lipid strips were blocked in 3% fatty-acid-free BSA in TBS-T containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% Tween-20 for 1h at room temperature in the dark. After binding, membranes were incubated at 4°C with gentle agitation in the same solution containing 1μg/ml GST, GST-PX (phosphoinositide-binding structural domain-positive control) or GST-TG2. The membranes were washed three times in TBS-T and then incubated for 1h with anti-GST antibody followed by immunoblotting.

Time- lapse video

For time lapse video macrophages were labeled with CMTMR as above and were exposed to unlabeled apoptotic thymocytes in a 6 target:1 macrophage ratio. Time-lapse movies were made using both fluorescence emission (in the standard rhodamine channel) and transmission of green (543 nm) light in a Zeiss LSM510 confocal laser scanning microscope equipped with a 40x/1.2NA water immersion objective. 1024x1024 pixel images were taken every 10 sec at 140 nm/pixel resolution. Regions of interest were extracted and exported to mpg format with 16 frames/s speed, yielding a compressed jpg video 160 times the actual speed of the process of phagocytosis.

RESULTS

The efficiency of apoptotic cell and carboxylate-modified latex bead uptake by TG2$^{-/-}$ macrophages is lower compared to their wild type counterparts

Adding apoptotic thymocytes to macrophages derived from TG2$^{-/-}$ and wild type mice different phagocytotic efficiency was observed. For precise determination of phagocytosis macrophages and apoptotic cells were fluorescently stained**,** and either the number of engulfed cells was counted under confocal microscope or the percentage of macrophages engulfing apoptotic cells was determined by flow cytometry at various time points. During 1 hour period of phagocytosis approximately 85% of wild type (WT) macrophages engulfed at least one apoptotic cell. In contrast, 50% of TG2$^{-/-}$ cells were not able to engulf apoptotic cells at all, and the numbers of phagocytosed cells were much lower compared to WT counterparts.

2-μm carboxylate-modified latex beads were previously used to replace apoptotic cells in signaling studies. The clearance efficiency of these beads by TG2$^{-/-}$ macrophages was also impaired compared to WT macrophages as we seen in case of apoptotic cells.

Cell surface TG2 having intact guanine nucleotide binding pocket promotes the phagocytosis of apoptotic cells

Phagocytosis of apoptotic cells by wild type macrophages was not significantly affected by a competitive inhibitor of TG2

(monodansylcadaverine, 15 μM), suggesting that the crosslinking activity of TG2 was not required for phagocytic activity.

To determine which function or structure of TG2 participates in phagocytosis several TG2 mutants were generated. Adenovirus was used to deliver WT, cross-linking, fibronectin binding, GTP/GDP binding and cell surface secretion deficient TG2 mutants into $TG2^{-/-}$ peritoneal macrophages. Phagocytosis experiments after different virus infection showed that not the cross-linking activity of TG2 but the GTP/GDP binding conformation was necessary for proper phagocytosis process and that the TG2 enzyme had to be present on the cell surface. In addition, exogenously added human recombinant TG2 (2 μg/ml) also stimulated phagocytosis of apoptotic cells by $TG2^{-/-}$ macrophages (from 36.8±4.7% to 77.6±11.9, n=3, $p>0.002$) indicating that cell surface TG2 is likely required for the engulfment process.

Biotin-XX SSE labeling was used to tag cell surface proteins. 10% of the total TG2 expressed on the cell surface assayed by densitometry. Using biotin-labelling technique the cell surface appearance of TG2 was teste. The wild-type and both guanine nucleotide binding variants, but not the secretion mutant appeared on the cell surface suggesting that guanine nucleotide binding is required not for the cell surface transfer. Together, these data indicate that cell surface TG2 in guanine nucleotide bound conformation promotes phagocytosis of apoptotic cells.

TG2 is required for proper integrin β_3 signaling

Determining mRNA expression of various phagocytosis-related receptors by TaqMan assay we found that mRNA of integrin β3 was expressed at a higher level in $TG2^{-/-}$ cells. Since cell surface TG2 was reported be a coreceptor for integrin β_3, and integrin β_3 plays a central role in the uptake of apoptotic cells, we speculated that $TG2^{-/-}$ cells might upregulate integrin β_3 to compensate the loss of TG2. Towards this goal, we tested the functionality of the integrin β_3 signaling pathway in $TG2^{-/-}$ cells by determining the activation of RhoG and Rac. Loss of TG2 did not affect the total amount of Rac1, while RhoG levels were slightly elevated. Addition of beads to WT macrophages triggered activation of both RhoG and Rac1 detected by ELMO and PAK pull down assays, respectively. In the absence of TG2 RhoG and Rac1 could not be activated in detectable amounts following exposure to carboxylate-modified latex beads. These data indicate that the integrin β3 pathway is impaired in $TG2^{-/-}$ cells.

Examination of the engulfment mechanism

Rac cycles between the membrane and the cytosol. In its inactive status, Rac remains in the cytoplasm and becomes activated upon cell stimulation, when it is translocated to the membrane interact with downstream effectors.

In non-engulfing WT cells Rac1 was found in the perinuclear region, while in engulfing cells it concentrated at one pole of the macrophage. This pole was surrounded by well organized F-actin filaments. Seeing many sections we noticed that the uptake of the apoptotic cells occurred through one or two Rac1 rich poles, which

acted as an effective engulfing gate in the macrophage. The apoptotic cells taken up by one portal remain separated from those apoptotic cells, which were taken up by the other portal.

In contrast, in $TG2^{-/-}$ cells most of the Rac1 was found in the perinuclear region in both non-engulfing and engulfing macrophages implying that in the absence of TG2 Rac1 cannot be fully activated and concentrated around the apoptotic cells and the Rac rich pole could not be formed. In addition, due to the improper signaling an abnormal F-actin polymerisation was detected at the apoptotic cell binding sites. Because in the absence of TG2 an efficient engulfing pole could not be built up, $TG2^{-/-}$ macrophages tend to take up the apoptotic cell at different sites.

In line with the mRNA data, the expression of integrin β3 protein level was elevated in $TG2^{-/-}$ cells as compared to their WT counterparts. Despite of the increased protein levels, we did not detect any significant change in the cell surface distribution of integrin β_3 receptors in the absence of TG2 on non-engulfing macrophages as compared to WT cells. During phagocytosis, however, WT cells concentrated integrin β_3 at one pole of the cell, where the uptake of the apoptotic cells occurred, while $TG2^{-/-}$ macrophages were unable to do so.

The lack of integrin β3 accumulation around the apoptotic cells in $TG2^{-/-}$ macrophages seems to be specific for integrin β_3, since accumulation of another phagocytic receptor, LRP-1, which was also shown to interact with TG2 was not affected. These data indicate that TG2 is required for the recognition of apoptotic cells by the MFG-

E8/integrin β_3 ligand/receptor complex and for its consequent accumulation around the apoptotic cell. In the absence of integrin β_3 signaling the initiation of the engulfing centre, which will efficiently take up the additional apoptotic cells, is also impaired.

Study of interaction between TG2 and milk fat globulin-8 (MFG-E8)

We tested possible molecular interactions of TG2 besides binding to integrin β_3. MFG-E8 was shown previously to bridge integrin β_3 to phosphatidylserine on apoptotic cells, we tested whether TG2 can also recognize phosphatidylserine using membrane strips spotted with various phospholipids. TG2 did not bind to phosphatidylserine on the membrane strips indicating that while MFG-E8 acts as a bridging molecule between phosphatidylserine and integrin β_3, TG2 does not.

Since in cell adhesion TG2 was shown to enhance integrin β_3 signaling by forming ternary complexes with the integrin β_3 ligand fibronectin and integrin β_3 via binding to both, we decided to check whether TG2 can similarly interact with MFG-E8, the phagocytic integrin β_3 ligand, by using surface plasmon resonance technique. Sensograms obtained at 5 different MFG-E8 concentrations are indicating interaction of MFG-E8 with TG2 in a concentration range of 5 to 200 nM. Fitting the sensograms according to a simple 1:1 binding model the association constant (K_a= 1.86 x 10^8) was determined for this interaction. As a comparison, the association constant (K_a= 1.00 x10^7) for the binding of TG2 to fibronectin was also determined. These data

imply that the MFG-E8/TG2 complex is reasonably stable and suggest that this interaction may also occur under physiological conditions.

Examination of phagocytic activity and morphology of macrophages from a TG2$^{-/-}$ subline

To generate enough TG2$^{-/-}$ macrophages for the phagocytosis experiments, we crossed TG2$^{-/-}$ mice with each other through several generations and noticed that after a one year period of crossing macrophages from these mice had a lower phagocytic activity than macrophages from those TG2$^{-/-}$ mice which were originated from crossing heterozygous mice. While following exposure to apoptotic cells for 1 hour 45±12% of TG2$^{-/-}$ macrophages were able to engulf at least one apoptotic cell, only 25±8% ($p < 0.05$) of the macrophages from this subline had phagocytic activity. When we looked by confocal microscopy at the number of apoptotic cells engulfed by macrophages from this subline predominantly one single apoptotic cell was engulfed.

Not only the phagocytic capacity, but the appearance of the macrophages from this subline has also dramatically changed. While non-engulfing TG2$^{+/+}$ and TG2$^{-/-}$ cells had fibroblastoid forms, and Rac1 was located in the cytoplasm in the perinuclear region, in non-engulfing macrophages of the TG2 subline a clear recruitment of Rac1 to the plasma membrane could be detected.

Study of integrin β_3 signaling in the TG2$^{-/-}$ subline

The absolute levels of Rac1 have not changed in these TG2$^{-/-}$ macrophages as compared to wild-type or to the average TG2$^{-/-}$

macrophages. However, the amount of active Rac1 was significantly elevated. Integrin β_3 signaling can be coupled to Rac1 activation via activating RhoG. However, while RhoG-GTP levels were not elevated in the $TG2^{-/-}$ macrophages in the macrophages from the $TG2^{-/-}$ subline an elevated Rho-GTP level was detected.

When macrophages from the TG2 subline were exposed to carboxylate-modified latex beads active RhoG level be further elevated but the elevated Rac1-GTP levels could not be increased anymore. In context with these observations, when these macrophages were exposed to apoptotic cells, Rac1 remained recruited all over the periphery and did not accumulate around the apoptotic cells.

Concomitantly, much higher levels of integrin β_3 were found on the cell surface of these macrophages. When these macrophages were kept in the presence of soluble vitronectin, which competes with the binding of the immobilized integrin β_3 ligands, the levels of active Rac1 were decreased proving that the elevated amount of Rac1-GTP levels are indeed a consequence of the enhanced integrin β_3 levels and signaling in these cells.

Although receptor level of integrin β_3 increased all around the macrophage, macrophages of this TG2 subline also did not concentrate integrin β_3 around the apoptotic cells. Increased levels of integrin β_3 leads to the elevation of active RhoG, whereby this TG2 subline compensates the loss of TG2 in the phagocytosis signaling at least at the level of RhoG activation.

Over-expression of wild-type Rac1 can compensate the defect of phagocytosis in macrophages of the TG2$^{-/-}$ subline

To test whether providing of additional Rac1 molecules could overcome the defect in integrin β_3 signaling, macrophages from the TG2$^{-/-}$ subline were transfected with wild type or constitutively active Rac1 molecules by adenoviral gene delivery, and their phagocytosis was detected by FACS analysis. Addition of constitutively active Rac1 completely inhibited the phagocytosis of apoptotic cells, indicating that switching on and off of Rac1 is required for proper phagocytosis of apoptotic cells. However, when these cells were transfected with wild type Rac1, Rac1 accumulation could be detected around the apoptotic cells to form the efficient phagocytic portal. Consequently the phagocytosis rate of the Rac1-transfected cells reached that of the wild type cells (75±12 % and 82±7% for wild-type and TG2$^{-/-}$ /integrin β_3 high macrophages, respectively). Not only the percentage of the engulfing macrophages has increased following Rac1 transfection, but Rac1-transfected TG2$^{-/-}$ macrophages also efficiently took up apoptotic cells, though their enhanced motility has remained.

Apoptotic cell-induced formation of 3-phosphoinositides is impaired in TG2 null macrophages

Activation of phosphatidylinositol-3-OH kinase (PI-3kinase) is required for proper phagocytosis, and both DOCK180 and ELMO contains recognition domains for 3-phosphoinositides for proper membrane localization and thus directing Rac1 activation. To detect formation of 3-phosphoinositides during the phagocytosis of apoptotic

cells both wild-type and $TG2^{-/-}$ macrophages were transfected by PLCδ-PHD-GFP. Apoptotic cell recognition induced formation of 3-phosphoinositides around the apoptotic cells in wild-type macrophages, while this induction was impaired in $TG2^{-/-}$ macrophages indicating that not only the activation of RhoG, but proper activation of the phosphatidylinositol-3-OH kinase is also under the control of TG2.

DISCUSSION

In this study we investigated phagocytosis of apoptotic cells by peritoneal macrophages *in vitro* to understand how the TG2 contributes in macrophages to proper engulfment of apoptotic cells besides activating TGF-β. To clarify the role of TG2 we used peritoneal macrophages derived from mice lacking $TG2^{-/-}$ and wild type macrophages in an *in vitro* phagocytosis model.

The engulfment of apoptotic cells is regulated by a highly redundant system of receptors, bridging molecules and 'eat me' signals. The complexity of the system is reflected by the term: 'engulfment synapse', used to describe the interaction between a phagocytic cell and its target. Recruitment of integrins to the apoptotic cells to form phagocytic cup suggests that integrins are one of the components of the ‘‘engulfment synapse’’.

In our study we described for the first time that in contrast to the present view of stochastic uptake, phagocytosis of apoptotic cells occurs through one or two engulfing gates within the macrophages, which will take up the apoptotic cells. We propose the efficient engulfing centers are characterized by phagocytic receptor and Rac1 accumulation following interaction with the apoptotic cells at a pole of the macrophage, but their place or number is very likely determined by yet unknown molecules. Accumulation and activation of integrin β_3 around the apoptotic cell is part of the initiation of such a centre, and occurs with the contribution of cell surface TG2, at least in thioglycollate elicited peritoneal macrophages. In the absence of TG2 the formation of phagocyte portal is less efficient, and even those

portals, which are formed, take up the apoptotic cells at a much slower rate. As a result, the uptake of apoptotic cells becomes slow and random.

As an explanation for the slower phagocytosis, we found an altered integrin signaling in $TG2^{-/-}$ macrophages. Neither activation of RhoG, nor that of Rac1 was found proper in $TG2^{-/-}$ macrophages. As a result Rac1 could not accumulate properly at a pole of the macrophage and the F-actin structure was also disorganized. To try and compensate the loss of TG2 macrophages increased the expression of integrin β_3.

TG2 is able to form a complex with MFG-E8, a known bridging molecule for integrin β_3. According to our results the binding affinity of TG2 to MFG-E8 was higher than that to fibronectin (Fn), a bridging molecule of integrin β_3 to the extracellular matrix (ECM). Integrins are relatively low affinity receptors for ECM proteins, including Fn. In contrast, TG2 binds with high affinity to Fn, and forms stable complexes with integrins. As a result, TG2 promotes integrin β_3 signaling in cell adhesion via stabilizing the integrin-Fn interaction. Our finding that MFG-E8 is also a binding partner for integrin β_3 indicates that TG2 might similarly affect integrin signaling in the context of phagocytosis as it does on the context of cell adhesion or migration on Fn.

We found that to promote phagocytosis TG2 must have intact guanine nucleotide binding sites. According to our findings for the cell surface transfer of TG2 guanine nucleotide binding is not required, but Tyr-274 residues plays important role. Thus we propose that guanine nucleotide binding is required for TG2 to fulfill its phagocytic function

on the cell surface. This is in line with a previous observation, which demonstrated that GTP binding affects the effect of TG2 on integrin signaling. Guanine nucleotide binding conformation may allow TG2 to form complex with one or more extracellular matrix proteins, bridging molecule or external domains of cell surface receptor to induce rapid signaling events leading actin rearrangement and efficient engulfment. Thus we propose that guanine nucleotide binding of TG2 in the context of phagocytosis is also required for gaining the proper conformation for the interaction with MFG-E8 and integrin β_3. Since the engulfment process was not dependent on the transamidation activity of TG2, it is very likely that the interaction between TG2 and MFG-E8/integrin β3 complex occurs at its inactive conformational state.

Interestingly on the cell surface TG2 interacts also with another phagocytic receptor, LRP1, which is involved in the removal of TG2 from the cell surface. However, neither the levels, nor the association of LRP-1 with the phagocytic synapse was affected by the loss of TG2. Still based on our present results, we cannot exclude, that LRP-1 signaling is also affected by the loss of TG2.

While we were studying $TG2^{-/-}$ mice, we found a subline, in which the macrophages compensated the loss of TG2 by a much higher expression of integrin β_3 than the average $TG2^{-/-}$ macrophages. Integrin β_3 and RhoG signaling is involved not only in the phagocytosis of apoptotic cells, but also in cell motility. As a result, increased expression of integrin β_3 in these cells resulted in enhanced motility with high basal active RhoG and Rac1 levels. Concomitant with the elevated basal active Rac1 levels, in non-engulfing $TG2^{-/-}$/integrin β_3

high macrophages Rac1 was located mostly bound to the cellular membranes, while we found it in the cytosol in wild –type and in regular $TG2^{-/-}$ macrophages. When these macrophages were exposed to apoptotic cells, integrin β_3 remained evenly distributed on the cell surface, but, due to the enhanced expression a high density was detected around the apoptotic cells as well. In these cells addition of carboxylate-modified latex beads, unlike in regular $TG2^{-/-}$ macrophages, triggered the activation of RhoG, indicating that the elevated integrin β_3 levels were sufficient to overcome the defect caused by the loss of TG2 in the initiation phase of integrin β_3 signaling. Still activation of Rac1, a downstream target of RhoG, could not be observed, and the activation of PI-3kinase was impaired. Concomitantly, we could not detect a significant accumulation of Rac1 around the apoptotic cells, and the phagocytosis of apoptotic cells was more severely affected.

Though integrin β_3 and RhoG are involved in both cell motility and phagocytosis, TG2 is not equally required for both. While phagocytosis of apoptotic cells is enhanced, integrin-dependent migration on laminin for example is inhibited by TG2. Thus the loss of TG2 might not equally affect the phagocytic and motility signaling pathways, especially that of the activation of PI-3kinase, which seems to be independent of integrin signaling in the context of phagocytosis (Raymund Birge, personal communication). Moderate increase in integrin β_3 expression observed in $TG2^{-/-}$ macrophages is expected to enhance the impaired phagocytosis and to induce enhanced motility. However, if the levels of integrin β_3 increase further, the two pathways

might become competitive, integrin-induced motility winning by being too efficient in Rac1 activation and using up free Rac1. This is because total RhoG levels adapted to the changes in integrin β_3 expression and enhanced integrin signaling, but there were no alterations in the basal Rac1 levels. Increasing the free Rac level by transfection of Rac1 molecules, we could improve the phagocytosis, but transfection of constitutively active Rac1 completely inhibited the phagocytosis of apoptotic cells. This observation confirms that of *Nagaya* and suggests that dynamic switching on and off of Rac1 is required for proper phagocytosis. Macrophages transfected with wild type Rac were able to concentrate additional Rac1 around the apoptotic cells and engulfed apoptotic cells with a similar rate as wild-type macrophages.

These data provide a further proof for the hypothesis that the role of TG2 in phagocytosis is to provide efficient integrin β_3 signaling around the apoptotic cells (either by promoting integrin β_3 clustering in the phagocytic cup, or by enhancing the affinity of the receptor for its ligand MFG-E8/phosphatidylserine), which in the absence of TG2 can also be achieved by an enhanced receptor density. Our data, however, might also suggest that TG2 is required for additional signaling pathways involved in the activation of PI-3kinase. Activation of PI-3kinase is essential for proper phagocytosis, and both DOCK180 and ELMO contain recognition domains for 3-phosphoinositides for proper membrane localization and thus directing Rac1 activation. Based on our data, integrin signaling will not affect the number of phagocytic poles formed, but as it was suggested by others, it might be critical in the initiation of the formation of the cellular pole.

SUMMARY

The clearance of apoptotic cells by macrophages plays a crucial role in tissue repair, suppressing inflammation and regulating immune responses. Transglutaminase 2 (TG2) is a protein crosslinking enzyme with diverse biological functions. Among many others it acts as an integrin β_3 co-receptor. We have previously shown that in $TG2^{-/-}$ mice the *in vivo* clearance of apoptotic cells is defective leading to development of SLE like autoimmunity. This was partially related to a defect in TGF-β activation, as TGFβ released by macrophages digesting apoptotic cells promotes phagocytosis of apoptotic cells and inhibits inflammatory responses.

In the present work the role of TG2 was studied in details in the engulfment of apoptotic cells by macrophages. Here we report that TG2 promotes phagocytosis of apoptotic cells by acting on the macrophage cell surface in guanine nucleotide bound form. Besides being a binding partner for integrin β_3, a receptor known to mediate the uptake of apoptotic cells via activating Rac1, we also show that TG2 binds milk fat globule EGF-factor 8 (MFG-E8), a protein known to bridge integrin β_3 to phosphatidylserine on apoptotic cells. We report that in wild-type macrophages one or two engulfing portals are formed during phagocytosis of apoptotic cells that are characterized by accumulation of integrin β_3 and Rac1. In the absence of TG2, although the levels of integrin β_3 are enhanced, integrin β_3 and consequently Rac1 can not be concentrated and activated at one pole of the macrophage. The defect in the $\alpha_v\beta_3$ integrin signaling leads to an

abnormal actin cytoskelatal organization and the efficient engulfing gate is not formed. Together, our data indicate that TG2 is a new protein member of the phagocytic cup, which together with MFG-E8 is required for proper apoptotic cell recognition and integrin β_3 signaling.

In the present study we also describe a subline of $TG2^{-/-}$ mice, in which a compensatory increase in integrin β_3 expression, which resulted alone in a high receptor concentration around the apoptotic cells without the requirement for accumulation, the elevated integrin β_3 levels were sufficient to overcome the defect caused by the loss of TG2 in the initiation phase of integrin β_3 signaling, but a significant accumulation of Rac1 around the apoptotic cells did not occur, and the phagocytosis of apoptotic cells was more severely affected. The lack of Rac1 accumulation was partially related to a defect of PI-3-kinase activation. Our data provide a proof for the concept that the function of TG2 is to stabilize accumulated integrin β_3 concentration in the phagocytic cup.

PUBLICATION

Publications related to the thesis

Tóth, B., Garabuczi, E., Sarang, Z., Vereb, G., Vámosi, G., Aeschlimann, D., Blaskó, B., Bécsi, B., Erdõdi, F., Lacy-Hulbert, A., Zhang, A., Falasca, L., Birge, R.B., Balajthy, Z., Melino, G., Fésüs, L., Szondy, Z. (2009) Transglutaminase 2 is needed for the formation of an efficient phagocyte portal in macrophages engulfing apoptotic cells. J Immunol. **182**, 2084-92. IF:6.068

Tóth, B., Sarang, Z., Vereb, G., Zhang, A., Tanaka, S., Melino, G., Fésüs, L., Szondy, Z (2009) Over-expression of integrin beta3 can partially overcome the defect of integrin beta3 signaling in transglutaminase 2 null macrophages. Immunol. Lett. 2009 Jul 28. [Epub ahead of print]. IF: 2.858

Sarang, Z., **Tóth, B**., Balajthy, Z., Köröskényi, K., Garabuczi, E., Fésüs, L., Szondy, Z. (2009) Some lessons from the tissue transglutaminase knockout mouse. Amino Acids **36**, 625-31. IF: 4.132

Other publications

Tóth B, Ludányi K, Kiss I, Reichert U, Michel S, Fésüs L, Szondy Z. (2004) Retinoids induce Fas(CD95) ligand cell surface expression via RARgamma and nur77 in T cells. Eur. J. Immunol. **34**. 827-36. IF: 4.772

Kiss I, Rühl R, Szegezdi E, Fritzsche B, **Tóth B**, Pongrácz J, Perlmann T, Fésüs L, Szondy Z. (2008) Retinoid receptor-activating ligands are produced within the mouse thymus during postnatal development. Eur. J. Immunol. **38**, 147-55. IF: 4.865

First authored posters on international meetings

Tóth, B., Kis-Tóth, K., Szondy, Z.: Role of Fas ligand and nur77 in T cell apoptosis induced by retinoids .From transcription to physiology:Regulation of gene expression and protein. FEBS Summer School. Spetses, Grecce, 2003

Tóth, B., Kis-Tóth, K., Szondy, Z.: Role of Fas ligand and nur77 in T cell apoptosis induced by retinoids. 12th Euroconference on Apoptosis, Chania, Greece, 2004

Tóth, B., Sawatzky, D.A., Fésüs, L., Szondy, Z: Role of tissue transglutaminase in process of phagocytosis. 30th FEBS Congress and 9th IUBMB Conference, Budapest, 2005

Tóth, B., Sarang, Z., Fésüs, L., Szondy, Z: Phagocytosis and proinflammatory cytokine production of TG2-/- macrophages. 8th International Conference on Protein Crosslinking and Transglutaminases (PCL8), Lübeck, Germany, 2005

Tóth, B., Sawatzky, D.A., Fésüs, L., Szondy, Z: Role of tissue transglutaminase in the phagocytosis. 13th Euroconference on Apoptosis, Budapest, 2005

Tóth B., Aeschlimann D., Fésüs L., Szondy Z.: Role of tissue transglutaminase in the process of phagoytosis of apoptotic cells. 14th Euroconference on Apoptosis, Chia, Sardinia, Italy, 2006

Tóth, B., Garabuczi, É., Vereb, G., Falasca, L., Fésüs, L., Szondy, Z.: Role of tissue transglutaminase in apoptophagocytosis program I: The macrophage side. 9th International Conference On Transglutaminases And Protein Crosslinking (PLC9), Marrakech, Morocco, 2007

Presentation:

Tóth B., Aeschlimann D., Fésüs L., Szondy Z.: Role of tissue transglutaminase (TG2) in the phagoytosis of apoptotic cells; IADR PEF congress, Dublin, Ireland, 2006