THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Transglutaminase 2 as an essential regulatory factor of neutrophil granulocyte differentiation

Potential contribution in retinoic acid syndrome

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DEBRECEN, 2010

INTRODUCTION

Transglutaminase 2

Transglutaminase 2 (TG2) is a thiol- and Ca^{2+}-dependent acyl-transferase that catalyze the formation of covalent cross-links between γ-glutamyl groups of peptide-bound glutamine residues and the ε-lysyl group of lysine residues in certain proteins. The reaction results in a covalent post-translational modification by establishing the γ-glutamyl-ε-lysyl cross-linkage between the two substrate proteins.

TG2 consists of 687 amino acid residues, which compose four distinct domains: an N-terminal β-sandwich, a catalytic core and two C-terminal β-barrel domains. The catalytic core domain carries the catalytic triad (Cys277-His335-Asp358) for the calcium-dependent acyl-transfer reaction, and it is also responsible for the protein disulfide isomerase, ATPase and a protein kinase activities. The core domain also contains a BH3 motif of the Bcl-2 protein family and one of two predicted nuclear localization signals (NLS1). Based on these findings TG2 may interact with the pro-apoptotic protein Bax and sensitize cells toward apoptosis and is also able to be transported into the nucleus by importin-α3 where it can modify proteins involved in gene expression regulation (e.g. histones or retinoblastoma). The first β-barrel domain is responsible for the GDP-binding of TG2. In the liver TG2 is able to act as a G-protein, it couples to α1-adrenergic receptor, activates phospholipase C (PLCδ1) and therefore it takes part in signal transduction. The GDP-bound enzyme forms a closed conformer contrary to the calcium-bound form, suggesting that the transition from the GTPase activity to the acyl-transferase one is accompanied by a large radical conformation change involving the whole structure of the protein. The second β-barrel domain carries the second predicted nuclear localization signal (NLS2) and the interaction site for phospholipase C is also localized at the C-terminal end of this domain.

There are different cells or tissues that constitutively express TG2 (such as mesangial or interstitial cells, thymic subcapsular epithelium, colonic pericryptal fibroblasts), while in others it seems to be induced either by external stimuli (epithelium of the female breast) or as part of their differentiation/maturation program (developing nephrons, enterocytes of the small intestine). Subcellular localization of the enzyme is also diverse, since inside the cell it was detected in the cytosol, in the mitochondrion and in the nucleus. TG2 can translocate to the plasma membrane in a 1:1 complex with β integrin. In the plasma membrane, TG2's association with integrins on the cell surface and its high affinity for fibronectin promote cell attachment to the proteins of the extracellular matrix and the transduction of external signals. Although its detailed externalization mechanism is unclear so far, the enzyme resides in the extracellular matrix either freely, coupled to ECM macromolecules and plays role in the modeling of the ECM.

The number of potential substrate proteins for TG2 is close to 150. Nevertheless, it is evident that a huge number of TG2 substrate proteins are involved in cell motility (actin, myosin, troponin, b-tubulin, tau, Rho A), in the interaction of cells with extracellular matrix structures (fibronectin, fibrinogen, vitronectin,

osteopontin, nidogen, laminin), and in key steps of energetic intermediate metabolism (aldolase A, glyceraldehyde-3-phosphate dehydrogenase, phosphorylase kinase).

TG2 in the function of the immune system

TG2 is widely present in immune cells and acts either as an important mediator of the differentiation of these cells or as a direct participant of their immune function. An increase in TG2 induction and activity seems to be a general phenomenon accompanying the differentiation and functional maturation of macrophages. TG2 is involved in the process of receptor-mediated endocytosis and also in the elimination of apoptotic cells by phagocytosis.

TG2 is involved in the induction of mouse alveolar macrophage fusion and in the conversion of latent transforming growth factor-beta (LTGF-beta) to active TGF-β. Cell surface TG2 serves as an integrin-associated adhesion receptor that might be involved in extravasation and migration of monocytic/macrophage cells into tissues.

TG2 was also expressed by duodenal CD11c(+) dendritic cells (DC) and externalized to their surface with a possible role in T cell activation in the celiac lesion. Peripheral blood monocyte-derived dendritic cells (iDC) contain large amounts of TG2 and it is also detectable on the surface of these cells. It was described that TG2 on the surface of the antigen presenting DC can be catalytically active; hence it might directly be involved in the deamidation of gliadin peptides promoting the development of celiac disease. The inhibition of the enzyme cross-linking activity led to impairment of DC function highlighted by wide phenotypic changes associated with a reduced production of cytokines (IL-10, IL-12) after LPS treatment and a lower ability to induce IFN-γ production by naïve T cells. *In vivo* analysis of DCs obtained from TG2(-/-) mice confirmed that the enzyme ablation leads to an impairment of DC maturation and their reduced responsiveness to LPS treatment. Taken together, these data suggest that TG2 is an important element of DC differentiation in response to LPS and could be a candidate target for treating endotoxin-induced sepsis.

The role of transglutaminase 2 in polymorphonuclear granulocytes was examined less extensively. However, its presence was described and its cross-linking-dependent role in the zymosan-induced superoxide production was also shown. TG2 was found to be involved in the induction of IL-1β in human peripheral blood-derived neutrophils in response to *Anaplasma phagocytophila*. Furthermore, it was demonstrated that TG2 can be induced in neutrophil granulocytes by granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment. It was also revealed in a study on rat basophilic leukemia cells that antigen stimulation induced expression and activity of TG2 by activation of NF-kB leads to increased production of reactive oxygen species, expression of prostaglandin E2 synthase (PGE2 synthase) and was responsible for increased secretion of prostaglandin E2. These findings suggest that increased expression of TG2 is closely associated to the inflammatory phenotype of polymorphonuclear granulocytes, since there are independent experiments showing that superoxide or inflammatory cytokine production of these cells –

which are hallmarks of the maturity of polymorphonuclear cells –depends on the presence and cross-linking activity of TG2.

Microglia also expresses TG2 and it is further inducible by LPS treatment and may contribute to brain inflammation mediated by nitric oxide. In lymphocytes TG2 expression and activity was demonstrated with a possible role in their proliferation.

TG2 in the regulation of the gene expression

There is ample evidence that TG2 may influence effectors of gene expression (histones, transcription factors etc.) or components of signaling pathways, which in turn impact on transcription. TG2 is able to cross-link and achieve the polymerization of retinoblastoma protein (pRb), which is paralleled by the rapid disappearance of E2F-1. It is also able to rescue the pRb from caspase-induced degradation in a transamidation-dependent manner. Therefore, according to these findings TG2 may contribute to the cell cycle regulation at gene expression level. In BV-2 microglia cell line TG2 catalyses the polymerization of the inhibitory subunit alpha of NF-κB (I-κBα). Polymerization leads to the dissociation of NF-κB and its translocation into the nucleus, where it is capable of up-regulating a host of inflammatory genes, including inducible nitric-oxide synthase and TNF-α. Similar activation of NF-κB was observed in doxorubicin-resistant breast cancer cell line, where again the TG2 was found to be responsible for the constitutive activation of the transcription factor, hereby for the maintenance of tumor progression.

Direct effect of TG2 on a transcription factor was observed in case of Sp1 inactivation. Ethanol induced apoptosis in hepatic cells enhances activity and nuclear accumulation of TG2. Nuclear TG2 is able to directly cross-link and inactivate the Sp1 transcription factor. As a consequence, reduced expression of the Sp1-responsive gene, c-Met and a subsequent apoptosis was observed.

Histones are also excellent substrates for the cross-linking activity of TG2. Therefore, TG2 may supply a possible tool for histone modifications suggesting a role for the enzyme in the regulation of chromatin structure and function.

In Huntington's disease the normal expression of cytochrome c and of its coactivator, peroxisome proliferator activated receptor-gamma coactivator-1 alpha (PGC-1α) is suppressed by TG2 due to a direct bound to proximal promoter of the cytochrome c gene and to the coding region of PGC-1α. This function of TG2 was Ca^{2+}-dependent and inhibited by a peptide-based specific irreversible cross-linking inhibitor of TG2. This result serves provides that TG2 is able to function as a selective corepressor of transcription.

Acute promyelocytic leukemia (APL)

Acute promyelocytic leukemia – a distinct subtype of acute myeloid leukemia (AML) – is characterized by a chromosomal translocation between the long arms of chromosomes 15 and 17 (t[15;17]). Translocation fuses the promyelocytic leukemia protein (PML) and retinoic acid receptor α (RAR-α), resulting in the synthesis of a fusion transcript (PML/RAR-α or RAR-α/PML).

In the process of differentiation of myeloid precursors towards neutrophil granulocytes retinoic acid induces extraordinary gene expression changes by the activation of RARα receptor. The complex transcriptional remodeling includes transcription factors, chromatin-modifying factors, cell cycle regulators, as well as regulators of apoptosis, stress response, metabolism and protein synthesis. Some of these differentially expressed genes may be directly involved in mediating terminal granulocytic differentiation, whereas others may merely reflect the biochemical and functional characteristics of mature granulocytes.

In case of PML/RARα mutation retinoic acid is unable to bind to the RARα and to induce terminal differentiation in physiological concentration. The fusion product acts as a dominant negative of both RARα and PML by forming homodimers, recruiting corepressors, and inhibiting expression of target genes necessary for granulocytic differentiation. Furthermore, the PML-RARα product may also inhibit the normal function of the protein as a tumor suppressor and therefore acts as a dominant negative against proteins.

Pharmacological concentration of RA leads to a conformation change of the multifunctional molecule complex around PML-RARα. Corepessors are released, normal regulation of RARα-responsive genes is restored, and hence, terminal differentiation and consequent apoptosis of APL cells is induced. Treatment of APL patients with retinoic acid achieves complete remission rates of up to 90% to 95% and is to date the most successful example of differentiation therapy.

Although ATRA is generally well tolerated, 2.5% to 31% of the APL patients who receive induction therapy develop the potentially lethal retinoic acid or differentiation syndrome (RAS or DS) as a side effect of ATRA-treatment. RAS is characterized by unexplained fever, weight gain, respiratory distress, interstitial pulmonary infiltrates, pleural and pericardial effusions, episodic hypotension, and acute renal failure.

In RAS an excessive systemic inflammatory response is observed, which can result in massive tissue infiltration of differentiating APL cells. The lung is one of the most relevant target organs. The initial migration of APL cells to the lung seems to be triggered by alveolar chemokine secretion. ATRA is able to significantly increase the production of specific chemokines (CCL2,-3,-22,-24 and CXCL8) by alveolar epithelial cells. Since upon ATRA-treatment APL cells also produce these chemokines, migrated cells into the lung further facilitate the infiltration of inflammatory cells into this organ. Therefore, production of chemokines by alveolar epithelial cells and differentiating APL cells may be important for the development of RAS.

MATERIALS AND METHODS

Cell culture

NB4 cells were cultured in RPMI 1640 and the 293FT packaging cell line was maintained in DMEM. Both media were supplemented with (10% v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Differentiation of NB4 was induced at 1 x 10^5 cells/mL with 1 µM ATRA.

Western blot analysis of TG2

Untreated and ATRA-treated NB4 cells were lysed in 250 mM sucrose, 1 mM DTT, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM PIPES, pH 7.4, 1 mM PMSF , 0.1% NP-40 at 4°C and homogenized with 8 to 10 strokes in a dounce homogenizer. Cytoplasmic and nuclear fractions were separated by centrifugation at 1100g for 15 minutes at 4°C. Cytosol was centrifuged at 13400g for 15 minutes at least 3 times to remove the remaining nuclei. The nuclei lysed in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM β-MEA, 0.1% Triton X-100, 0.5 mM PMSF. Lysates containing 2 mg/mL protein were mixed with equal volumes of gel loading buffer (0.125 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.02% bromophenol blue) and boiled for 10 minutes. Protein (25 µg) was electrophoresed on 8% SDS–polyacrylamide gels and electroblotted onto PVDF membrane. TG2 was detected with the CUB7402 monoclonal antibody.

Crosslinking activity assay of TG2

TG2 activity was measured in a reaction mixture containing 50 µL (2 mg/mL) crude cell fraction homogenate, 10 µL N, N-dimethylcasein (40 mg/mL), 20 µL [1,4(n)-3H] putrescine (30 Ci[37 MBq]/mmol), 10 µL 250 mM Tris-HCl, pH 7.5, containing 150 mM β-MEA, and 10 µL $CaCl_2$ (50 mM). The reaction was initiated by the addition of $CaCl_2$, incubated at 37°C for 5 minutes, and then 25 µL of sample dropped on filter paper, precipitated in cold trichloroacetic acid, and washed intensively with 10% and 5% TCA and ethanol. The radioactivity on the filter paper was measured in a liquid scintillation β-counter.

Immunolabeling of cells

Cells were fixed with (1) 4% paraformaldehyde in HEPES (4°C; 10 minutes), (2) 8% paraformaldehyde in HEPES (4°C; 50 minutes); (3) 4% paraformaldehyde in HEPES (4°C; 20 minutes), (4) 2% paraformaldehyde in PBS (15 minutes), and then (5) methanol (-20°C; 20 minutes). After fixation, cells were incubated in 25 mM glycine in PBS (20 minutes); permeabilized with 0.1% Triton X-100 in PBS (20 minutes); washed 5 x 20 minutes in TTBS; blocked with TTBS, 5% BSA, or milk powder, pH 7.4 (20 minutes); incubated (2 hours) with mouse monoclonal antibody against TG2; washed 5 x 30 minutes in TTBS; incubated with Alexa Fluor 633 goat anti–mouse IgG (1 hour); washed 5 x 30 minutes in TTBS;

and then rinsed 3 times with PBS before coverslips were mounted in Mowiol. Images were collected using a Zeiss LSM 510 confocal microscope. PI and Alexa Fluor 633 were excited with 543-nm and a 633-nm HeNe lasers, and emission was detected through 560-610 nm BP, and 650 nm LP filters, respectively.

Measurement of Nε-(γ-glutamyl)-lysine

The amount of the Nε-(γ-glutamyl)-lysine cross-link was measured in homogenized samples. Briefly, the protein content of cytosolic and nuclear cell fractions was precipitated and lyophilized. After rehydration, extensive enzymatic digestion, and derivatization with phenylisothiocyanate the obtained Nε-(γ-glutamyl)-lysine isodipeptide derivative was separated on a cation exchange resin and then on a silica HPLC column, and finally, quantified after reverse-phase HPLC.

In situ labeling of TG2 activity

Cells were labeled with 6 mM 5-(biotinamido)-pentylamine for 12 hours, harvested, and fractionated into nuclear and cytosolic fractions. To visualize the proteins into which the 5-(biotinamido)-pentylamine had been incorporated, samples (25 μg protein) were electrophoresed on 8% SDS–polyacrylamide gels, transferred to PVDF membrane, and detected with horseradish peroxidase–conjugated streptavidin.

Determination of superoxide anion production

10^6 cells were mixed with 0.5 mM isoluminol or luminol in modified Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, 1 mM $CaCl_2$, and 1.5 mM $MgCl_2$; pH 7.3). The samples were kept for 5 minutes at 37°C and activated by addition of 50 nM PMA. Mouse peritoneal polymorphonuclear neutrophils' O^{2-} production was measured by a chemiluminescence assay from 10^5 cells using 100 μM L-012 dye. After measuring the background signal 50 nM PMA was added and incubated for 5 minutes. Chemiluminescence was counted in luminometer at intervals of 10 seconds.

Preparation of peritoneal neutrophils from mice

Wild-type and TG2-deficient mice were injected intraperitoneally with 1 mL 10% yeast extract. 4 days later the peritoneal cavities of the mice was lavaged with 3 mL RPMI-1640. Peritoneal cells were centrifuged and resuspended in RPMI-1640 culture medium. Granulocytes were allowed to adhere for an half hour and nonadherent cells were removed by washing.

Virus production and generation of stable TG2 knockdown NB4 cell line

A set of 5 anti human TG2 short hairpin RNA expressing lentiviral plasmids (pLKO.1-puro) was purchased from Sigma. As a negative control a non-target shRNA vector was used (Sigma) which expresses a shRNA sequence containing 4 base pair mismatches to any known human gene. 293FT cells were transfected at 60-70% confluence in 75 cm^2 cell culture flasks. 3 µg of the antiTG2 shRNA plasmid

(or non-target shRNA control plasmid) and 9 µg of ViraPower Packaging Mix were delivered into the packaging cells per flask with Lipofectamine 2000 according to the manual. The medium was replaced after 12 hrs and medium containing virus particles was collected on the second and the third days after transfection, filtered through 0.45-µm-pore-size cellulose acetate filters. For concentration viral supernatants were ultracentrifuged at 100 000 g for 2 hours at 4 °C. Pellets were resuspended in 200 µl sterile PBS, aliquoted and stored at -70 °C. Virus titers were determined by cell culture titration; 10-fold serial dilutions of the concentrated viruses were used to infect 10^5 HeLa cells in a six-well plate. After 24 h transduced cells were selected and expanded in the presence of 5 µg/mL puromycin. 8-10 day old colonies were counted and virus titers were calculated by multiplying the numbers of the colonies by the dilution.

For NB4 transduction 2 x 10^4 cells were transduced with TG2 shRNA1-5 or non-target shRNA carrying viral vectors at a MOI of 1.0. 24-36 hrs later stable transduced cells were selected by the administration of puromycin at a final concentration of 5 µg/mL. Efficiency of TG2 gene silencing was determined at different time points by real-time Q-PCR and by Western blot analysis after induction of TG2 by ATRA.

Microarray analysis

Differentiation of NB4 was induced at 1 x 10^5 cells/mL with1 µM ATRA and cells were harvested at 0, 48 or 72 hrs thereafter. Total RNA from 10^{10} cells was isolated using the RNeasy kit. Experiments were performed in biological triplicates. Further processing and labeling, hybridization to GeneChip Human Gene 1.0 ST Arrays (Affymetrix), and scanning were conducted at the Microarray Core Facility of the EMBL (Heidelberg, Germany). Image files were imported to GeneSpring 10.1. Raw signal intensities were normalized per chip (to the 50th percentile) and per gene (to the median). To identify significantly regulated genes we selected probe sets that showed at least 2-fold up- or down-regulation by eliminating probe sets with a ratio of signal intensity between 0.5 and 2. Finally, we performed a t-test for each pair of probe sets and filtered for values of $p \leq 0.05$. The PANTHER software (www.pantherdb.org/tools/genexAnalysis.jsp) was used for functional classification of genes.

Real-time Q-PCR

Total RNA was isolated from cells using Trizol Reagent and reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit according to the manufacturers' instructions. RT Q-PCR was carried out on an ABI Prism 7900 using Taqman probes. Transcript levels were normalized to the level of cyclophilin D and gene expression was determined by the comparative cycle threshold (C_T) method.

Flow cytometry

To detect the surface expression of CD11c 8 x 10^5 cells were incubated with FITC-conjugated anti-human CD11c mAb or with anti-IgG1 mAb as isotype negative control for 2 hrs at 4°C in 1 % BSA-PBS. Cells were washed, fixed by 1% PFA and then analyzed by flow cytometry on a FACS Calibur. The geometric mean fluorescence of the FITC-positive cells was used to calculate CD11c surface expression.

For detection of phagocytic capacity, 10^5 three day-differentiated NB4 cells were allowed to ingest FITC-labeled *Listeria monocytogenes* or *Staphylococcus aureus* at a ratio of 1:50 (cells/bacteria) for two hrs at 37°C or at 4°C (to assess the aspecific sticking of bacteria to the phagocytes). After 2 hrs cells were put on ice, washed 2x with 5 volumes of ice cold PBS, fixed with 1% PFA and then analyzed by flow cytometry. The percentage of FITC-positive cells was used as a measure of phagocytosis. Cell cycle analysis by flow cytometry was carried out by propidium iodide staining after alcohol fixation.

Cell adhesion and migration

For the determination of cell adhesion, cells were differentiated for 48 and 72 hrs, than plated into 1% BSA coated culture dishes for 1 hour. Nonadherent cells were removed by washing, then the remaining cells were fixed with ice cold methanol:acetic acid (4:1), stained with hematoxylin for 5 minutes and counted. Migration assays were performed in a BD Matrigel Invasion Chamber using 200 nM fMLP, 10ng/mL IL-8 and 10% FBS as chemoattractants. 2.5 x 10^4 differentiated cells were allowed to migrate for 14 hrs. Chemoattractive effect of ATRA-treated wild-type, virus control and TG2 KD NB4 cell supernatants on white blood cells (WBCs) was evaluated using transwell polycarbonate inserts (6.5 mm diameter) with 5 µm pores and carried out as described previously.

CC chemokine level measurements

Supernatants of undifferentiated, 2 and 3 day ATRA-treated NB4 cells and their sublines were used for quantification of secreted CC chemokines. The concentration of CCL2 and 24 was measured using the Quantikine human ELISA kit, CCL3 and 22 were quantified with the RayBio human ELISA kit.

RESULTS

ATRA-induced differentiation of NB4 cell line towards neutrophil granulocyte is accompanied by the induction of TG2 and its translocation into the nucleus

We demonstrated that after 24 hrs of the ATRA-treatment TG2 is starting to be accumulated in the cytosol and it reaches its maximum protein level on the third day of the differentiation. TG2 was detected also in the nuclear fraction of the ATRA-treated NB4 cells, but its accumulation in the nucleus was observable only from the second day of the differentiation and peaked after 4-5 days. Protein crosslinking activity of TG2 in the two fractions was also determined by detecting the incorporation of [3H]putrescine into casein. The crosslinking activity of TG2 in the cytosol lysate increased continuously in accordance with the protein level of the enzyme. Interestingly, in the nucleus the activity of TG2 was also detectable; however its level lagged behind compared to the cytosolic form and calculated to total protein level. By confocal microscopy and immunostaining of permeabilized differentiated NB4 cells we also validated its present in the nucleus by double staining for DNA and TG2 on the sixth day of the differentiation and it was also revealed that TG2 colocalizes with the overall ultrastructural organization of the nucleus of differentiating NB4 cells. Increasing amount of the protein-bound Nε-(γ-glutamyl)-lysine cross-links in both the cytosolic and the nuclear fractions of differentiating cells suppose that accumulating TG2 is catalytically active. To evaluate whether ATRA-induced TG2 in NB4 is active on primary amines such as polyamines as well, in situ biotinamido-pentylamine ($BNPH_2$) incorporation assay was applied. On the fourth day of the differentiation high numbers of proteins were modified by $BNPH_2$ in the nucleus and the cytosol of intact cells. These results indicate that TG2 is present and active in the cytosol and as well as in the nucleus of the differentiating NB4 cells.

Effect of inhibition of TG2 activity on ATRA-induced differentiation of NB4

To evaluate the role of the cross-linking activity of TG2 in neutrophil differentiation we applied monodansylcadaverine (MDC), a nonspecific competitive inhibitor of transglutaminase during the whole time of the differentiation. On days 4 and 5 after MDC treatment, a significant decrease of TG2 activity and the cross-link content of cytosol and nucleus were observed, while the level of the TG2 expression did not change.

Superoxide (O_2^-) production is characteristic for neutrophil granulocytes and it is considered as reliable marker of differentiation of myeloid cells determined by the NBT reducing ability. Untreated, proliferating NB4 cells are not able to reduce NBT, but after 24 hrs of ATRA-treatment the proliferation rate of the cells starts to slow down and in line with this they develop the ability to reduce NBT and formazan deposits are observable intracellular. When the differentiation of NB4 cells were induced in the presence of MDC, significant reduction was observed in the NBT reducing capacity of NB4 cells during the course of the differentiation. These results suggest that cross-linking activity of TG2 is a constituent part of the ATRA-induced differentiation of these leukemic cells.

O_2^- production measured by chemiluminescence assay correlated well with the results obtained by NBT-assay, since MDC-treated differentiated NB4 cells produced significantly lower amount of superoxide both extra- and intracellular.

These results indicate that during the differentiation process TG2 with its cross-linking activity is necessary for the development and assembly of the NADPH-oxidase system which is one of the most important immunological features of neutrophil granulocytes, since it plays essential role in the killing of phagocytosed pathogens. A so called $gp91^{phox}$ protein, a subunit of the NADPH-oxidase system was described to be responsible for the lower production of superoxide in certain cases. Therefore, we determined the expression level of $gp91^{phox}$ by Northern blot and real-time QPCR. With both methods we detected a reduction in the expression of $gp91^{phox}$ mRNA level in case of MDC treated differentiated NB4 cells. According to these results the lower level of superoxide production in MDC-treated differentiating NB4 is caused by the lower expression of $gp91^{phox}$. These finding suggest that during the differentiation process active TG2 is required for the optimal generation of the NADPH-oxidase system and TG2 contributes to this process at gene expression level.

Role of TG2 in mouse neutrophils

In the aim of evaluation of the role of TG2 in neutrophil function in vivo, we compared NBT positivity and superoxide anion generation in neutrophils derived from TG2+/+ and TG2-/- mice. Neutrophils were isolated from peritoneal exudates of mice previously injected with yeast extract. In accordance with the NB4 cell culture model, neutrophils of TG2-/- mice showed a 10-fold decrease in NBT positivity compared to the cells derived from WT mice. The neutrophils of TG2-/- mice generated one order of magnitude less superoxide anion than the TG2+/+ neutrophils when they had been stimulated with PMA.

To ascertain whether the lower expression of $gp91^{phox}$ is responsible for the reduced superoxide production in murine neutrophils similarly to the human cells we determined the relative mRNA expression of this subunit of the NADPH-oxidase system by real-time QPCR. In case of neutrophils isolated from TG2-/- mice, we experienced significant decrease in the expression of $gp91^{phox}$. By Western blot analysis we managed to confirm that the reduction in $gp91^{phox}$ mRNA level in TG2-/- neutrophils leads to, indeed, a diminish in the protein amount of this protein.

shRNA-induced knocking down of TG2 delays the differentiation process of NB4 cells

To evaluate the role of TG2 in neutrophil granulocyte differentiation, the TG2 gene was stably silenced in NB4 cell line through RNA interference. NB4 cells were transduced by anti-TG2 shRNA expressing lentiviral vector (TG2-KD NB4). Non-targeting shRNA control vector was also used to create virus control NB4 cell line. NBT reducing capacity of wild-type and virus control NB4 cells increased in the same manner during the differentiation and reached maximum levels at 72-96 hrs. Differentiating TG2-KD NB4

cells were also able to reduce NBT but the number of NBT positive cells was significantly lower during the whole process of the differentiation than those in control and the virus control NB4 cells.

The expression of the CD11c differentiation marker was measured at 48 and 72 hrs of differentiation and its expression in TG2-KD NB4 cells was 3-fold lower than in the control or in the virus control NB4 cells at each day of differentiation. CD11c surface expression was also evaluated at the second and the third day of the differentiation by flow cytometric analysis, and it was further confirmed that while control virus infection did not influence CD11c surface expression, knockdown of TG2 lead to decreased expression of CD11c on the cell surface.

Comparison of gene expression profiling of differentiating control and TG2 knockdown NB4 cells

To determine the influence of TG2 on NB4 differentiation, gene expression changes induced by ATRA in control NB4 cells were determined during the differentiation process (on days 0, 2 and 3) and compared to those in TG2-KD NB4 cells**.** During the differentiation process we detected 340 up-regulated genes on day 2 and 559 on day 3, and among these up-regulated genes 307 showed at least 2-fold increase on both days of the sampling in NB4 control cells. As for the suppressed genes, 1281 genes showed decreased expression on day two and 1586 on day three. 1059 genes were found to be repressed on both days of differentiation in control NB4 cells. Remarkably, in differentiating TG2-KD NB4 cells there were less up-regulated genes, 254 on day 2, 344 on day 3 and 208 overlapped, than in control ATRA-treated NB4 cells. On the other hand, there were 1011 down-regulated genes on day 2, 1360 on day 3 and 808 overlapped in differentiating TG2-KD NB4 cells, as compared to control. These data suggest that TG2 has significant impact on ATRA-induced changes in gene expression in NB4 cells.

Affected genes derived from the wild-type NB4 differentiation were found to be significantly enriched in the following categories: 'immune function', 'cell cycle regulation', 'nucleotide metabolism' and 'protein metabolism'. These results reflect well the biological events occurring upon ATRA-treatment, since during the differentiation cell cycle arrest is induced which is accompanied by a significant decrease in nucleotide requirement and protein synthesis.

In TG2-KD NB4 cells, the gene ontological categories characteristic for differentiation of control NB4 cells, identified above, contained fewer genes and several of those subcategories were not significantly overrepresented at reduced TG2 level. These data clearly indicate that the induction of TG2 in NB4 cells contributes to the transcriptional remodeling, which launches the terminal differentiation program of these leukemic cells.

TG2 facilitates the transcriptional alterations induced by ATRA

We identified 313 genes that are affected by TG2 during the differentiation. From them 197 are also regulated by ATRA, showing that TG2 has a significant impact mainly on the expression of differentiation related genes. When we focused on the ATRA and TG2 regulated genes we demonstrated that the genes,

which are normally repressed by ATRA, remained at a high expression level in TG2-KD NB4 cells during the differentiation. These results indicate that the inhibitory effect of ATRA, in case of these genes is mediated by TG2. In case of up-regulated genes by ATRA, knocking down of TG2 leads to the lower induction of these genes. This means that TG2 has a facilitating role in the induction of certain genes characteristic to the differentiation process. Taking together, these results suggest that TG2 and ATRA have mainly synergistic effects during the course of differentiation.

TG2 knockdown NB4 cells retain cell proliferation potential

ATRA induces cell cycle arrest and terminal differentiation of NB4 cells by down-regulating genes, which are responsible for the extensive proliferation of these leukemic cells. In accordance with the higher expression of cell proliferation-related genes (such as E2F5, CDK6) in TG2-KD NB4 cells we detected higher proliferation ability of these cells. At 48 hrs there were approximately 27% more cells, and even at 72 hrs about 10% more proliferating cells, in culture of TG2-KD NB4 than in controls. Cell cycle distribution in NB4 cell lines revealed that when TG2 is silenced significantly more cells were present in S phase on the third day of differentiation.

Immune functions are compromised in differentiating TG-KD NB4

Based on the microarray data, several functional deficiencies in differentiating TG2-KD NB4 cells could be predicted. As many genes with reduced expression levels in TG2-KD NB4 cells belong to the 'Cell motility' or 'Cell adhesion' categories (i.e. integrins and selectins), we tested whether these expression changes appear at the functional level. Undifferentiated NB4 cells are floating in culture but after the administration of ATRA they start to adhere to plastic surfaces. Numbers of adherent cells were determined in the second and third day of the differentiation and a significant reduction in the adherence of TG2-KD NB4 cells was observed. Microscopic analysis also revealed that differentiating NB4 cells without TG2 are less spread, remain spherical and are only weekly attached to surface. We also tested whether down-regulation of TG2 expression would affect the migration and chemotaxis of differentiating NB4 cells in response to the chemoattractant IL-8 and/or fMLP and we observed a reduction in migration of TG2-KD NB4 cells.

As differentiated TG2-KD NB4 shows marked reduction in the expression of several phagocytosis-related genes, such as CD14, CD36, MERTK and others, we were interested to see whether phagocytic capacity was influenced by the reduced TG2 level. Approximately 1.5-fold and 2-fold decreases in phagocytosis of *Listeria monocytogenes* and *Staphylococcus aureus* were detected, respectively. Measurement of highly-reactive oxygen species (ROS) generation by neutrophils during activation of respiratory burst is of great importance to evaluate the bactericidal activity of neutrophils. TG2-KD NB4 cells generated approximately 2.5-fold less superoxide anion than controls in accordance with the lower expression of NCF2, a major component of neutrophil NADPH-oxidase system in TG2-KD cells.

Production of CC chemokines involved in the differentiation syndrome is restricted in TG2-KD NB4 cells upon ATRA-treatment

In the induction of the members of CC chemokine family microarray profiling showed that CCL2, 3, 22 and 24 were expressed significantly lower in TG2-KD cells at 48 and 72 hours after ATRA-stimulation. We confirmed their reduced expressions both by real-time Q-PCR and measuring their concentration in the culture fluid. These results reveal that the suppressed TG2 expression can restrict the ATRA-induced CC chemokine productions of APL cells. We could also verify that the lower production of chemokines in TG2-KD NB4 cells leads to reduced chemotaxis of peripheral white blood cells in a transwell system.

DISCUSSION

In our experiments, we have clarified some part of the role of TG2 in the ATRA-induced differentiation of the NB4 promyelocytic leukemia cell line at the level of the regulation of gene expression. Undifferentiated NB4 cells do not express TG2, but after the administration of ATRA TG2, as a direct target gene of RARα, is strongly induced. Accumulating TG2 is present in the cytosol of differentiating NB4 cell, and we have demonstrated that TG2 also enters into the nucleus. TG2 possesses cross-linking activity in both compartments, since increasing expression of TG2 is associated with the increased amount of protein-bound Nε-(γ-glutamyl)-lysine cross-linked content of the cells, and also the administrated 5-(biotinamido)-pentylamine is incorporated into cellular proteins. However, despite the extensive investigations on this field the intracellular activation of TG2 is still generally a dubious question due to the low level of intracellular Ca^{2+} (20 and 100 nM). Ca^{2+} is required for the activation of TG2 in millimolar concentrations according to *in vitro* assay, but cells allow increases of up to 500 nM following cellular activation. This range of permittable intracellular Ca^{2+} concentrations raises the question of how the intracellular TG2 is activated. In this study, we have not examined the changes of cytosolic Ca^{2+} concentration during the course of the differentiation, but as the induction of TG2 upon ATRA-treatment is strongly accompanied by the increase of the Nε-(γ-glutamyl)-lysine cross-linked content of the cells and the incorporation of 5-(biotinamido)-pentylamine – as monoamine substrate of transglutaminases – into cytosolic and nuclear proteins, we assumed the activation of the enzyme. Treatment of differentiating NB4 cells with MDC, a competitive cross-linking inhibitor of TG2, provides further evidence for the presence of active TG2 in the differentiating cells, since the accumulation of cross-linked content can be diminished by using the inhibitor. It was an interesting finding that inhibition of TG2 activity is accompanied by reduced NBT positivity of the cells. Since NBT positivity is considered to be a reliable marker of differentiation, we suspected a role for TG2 in the differentiation. Reduced production of superoxide anion in the presence of MDC in differentiated cells suggested a failure in the function of the NADPH-oxidase system. Further investigation revealed that at least one component of the complex, the $gp91^{phox}$ subunit is expressed at a lower level in MDC-treated differentiated NB4 cells. These findings led us to ascribe a regulatory role in the induction of $gp91^{phox}$ to TG2. Experiments on TG2 KO mice confirmed our hypothesis, since TG2-/- mouse neutrophils also show reduced NBT-positivity and superoxide production and they also express the $gp91^{phox}$ at a decreased level.

To clarify the general role of TG2 in the differentiation process of NB4 we generated stable TG2 knockdown NB4 cells using lentivirus-mediated gene silencing that allowed us to study the differentiation process at reduced TG2 expression, taking into account, that besides the numerous advantages of shRNA-mediated gene silencing, complete and sustained elimination of the expression of a particular gene cannot be achieved, which may attenuate the consequences of knocking down of TG2. Nevertheless, we managed

to reduce the level of TG2 during differentiation as it was confirmed by the determination of protein amount of TG2 by Western blot and by the measurement of its activity.

ATRA-treated TG2-KD NB4 cells lag behind significantly in differentiation as indicated by the decreased level of CD11c mRNA, its surface expression and the diminished NBT reducing capacity of these cells. The delayed differentiation of the knockdown cells is also revealed in several functional consequences such as decreased chemotaxis, adherence, phagocytic capacity, superoxide production and their sustained proliferative ability. TG2 has been implicated in various physiological phenomena, which can individually bear importance to the function of neutrophils. Impairment of neutrophil function by the reduction of TG2 could, in principle, result from the compromise of one or more of the aforementioned processes. The unexpected comprehensiveness of phenotypic changes in TG2-KD NB4, however, suggests that these do not represent isolated instances of interference with the molecular machinery of specific granulocyte tasks (phagocytosis, adherence, migration), rather a break-up of the blueprint for the granulocyte specific molecular apparatus as a whole. This must entail a principally unique regulatory effect of TG2, executed higher upstream during the course of the signaling events which induce NB4 cell differentiation.

The immediate effect of ATRA on gene transcription results in comprehensive reprogramming of the transcriptome. In the literature there is ample evidence that TG2 may influence effectors of gene expression or components of signaling pathways, which in turn impact on transcription as it was described in detail in the introduction. To ascertain whether TG2 may be implicated in ATRA-induced gene expression regulation in NB4 cells total gene expression profiling was carried out. So far a whole genome gene expression analysis of ATRA-treated NB4 cells has not been performed. Besides identifying several new ATRA-regulated genes we managed to confirm the changes in the expression of several markers of differentiation such as C/EBPβ and ε, the HLH family, the interferon regulatory factors (IRFs), STATs and SWI/SNF family of proteins (SMARCD). The complex modulator role of TG2 in the differentiation process affects the remodeling of gene expression profile in ATRA-induced differentiated NB4 cells. We identified at least 300 genes whose expression was dependent on the presence of TG2 at some level and it turned out that they were mostly ATRA-dependent as well. Genes affected by both differentiation (ATRA-dependent) and TG2 seem to be regulated in an opposite direction. In details, 88.1% of the genes down-regulated by ATRA remained at a higher level in TG2-KD cells and most genes induced by ATRA in control NB4 cells showed lower expression level in the TG2-KD one. These results raise a possible synergistic/additive effect of TG2 with regulatory role of ATRA.

Detailed analysis of the genes whose expression was modulated by TG2 revealed three important findings. First, among the genes down-regulated by ATRA but kept at high level in TG2-KD cells cell cycle and cell proliferation-related genes were significantly overrepresented (e.g. E2F5, CDK6). This result led us to assume that TG2-KD cells remain more proliferative for a longer time; therefore TG2 is necessary for the attenuation of cell proliferation. Indeed, TG2-KD cells have higher dividing capacity with more

cells in S-phase in the early stage of the differentiation program. However, it has to be noticed that we did not observe a difference in the survival capacity of differentiated TG2-KD cells as compared to the differentiated control NB4 cells during long-term culturing, or in the rate of apoptosis induced by arsenic-trioxide.

Second is that the genes, which are ATRA-induced but less expressed in TG2-KD cells during differentiation, mostly belong to the 'Immunity and defense' (Granulocyte-, Interferon- and Cytokine/Chemokine mediated immunity), the 'Cell motility' and the 'Cell adhesion' categories. In TG2-KD cells expression of key molecules involved in chemotaxis, phagocytic capacity and superoxide production are missing from the normally up-regulated genes in control ATRA-treated cell. One of these is the paxillin that is necessary for adhesion and motility of leukocytes. PAK1/p21protein (Cdc42/Rac)-activated kinase 1 is known for taking part in the regulation of chemotaxis, chemokine-induced cytoskeletal actin polymerization, and oxidative burst. We found that the expression of S100A8, a recently described pro-inflammatory protein expressed by phagocytes and implicated both in NADPH oxidase activation by interaction with NCF2/p67PHOX or transepithelial migration of neutrophils also remained at lower level in TG2-KD NB4 cells. These examples suggest that TG2 is required for the development of the full innate immune function of differentiating NB4 cells including their full inflammatory responsiveness.

Third is related to the role of TG2 in the process of inflammation. TG2 is implicated in the enhancement of inflammation since TG2 inhibitory peptide significantly decreased the production of inflammatory cytokines and neutrophil infiltration into lung in LPS-treated mice. Furthermore, TG2 knockout mice are partially resistant to LPS-elicited experimental septic shock with increased survival, a diminished inflammatory response and attenuated organ damage. The expression level of the CCL2 inflammatory mediator was consistently up-regulated in ATRA treated APL patients. The simultaneous expression of cytokines such as TNF-α, IL-1β, IL8 and CCL2 by ATRA treated leukemia cells, such as NB4 cells, may result in both increased binding to epithelial cells and chemotactic transmigration and thereby further accelerate tissue infiltration. Despite of the corticosteroid treatment a massive induction of CC chemokines (e.g. CCL2) appears in ATRA treated APL patients that might lead to the differentiation syndrome with excessive inflammatory response. Since knocking down of TG2 in NB4 cells is accompanied by reduced chemokine production (CCL2, 3, 22 and 24) upon ATRA-treatment and therefore leads to decreased development of chemoattractant capacity, we propose that, there is an essential role of TG2 in the regulation of inflammatory responsiveness and development of differentiation syndrome.

Based on these findings presented here, it may be a viable option to interfere with such a pathological condition through modulating TG2 level in the differentiating leukemic cells. Specific down-regulation of TG2 expression by gene silencing or using appropriate compounds may lead to gene-specific suppression of cytokine secretion in retinoic acid syndrome patients. Such a therapeutic approach may also work in other TG2-related in gain-of-function diseases like neurodegeneration, fibrosis, inflammation, cardiac failure, and celiac disease.

SUMMARY

Therapy of acute promyelocytic leukemia (APL) primarily consists of an *all-trans*-retinoic acid/ATRA-based treatment, which results in terminal differentiation of leukemic cells toward neutrophil granulocytes. However, this differentiation-inducing therapy is often accompanied by a severe hyper-inflammatory response in the lung as a potentially lethal side effect of ATRA-treatment, called retinoic acid syndrome (RAS). Administration of ATRA leads to massive changes in gene expression in APL cells, including down-regulation of cell proliferation related genes and induction of genes involved in immune functions of neutrophil granulocytes. One of the most induced genes by ATRA in APL NB4 cells is transglutaminase 2 (TG2).

We have demonstrated that transglutaminase 2, after its induction, partially translocates into the nucleus, associates to the chromatin and is able to modify nuclear proteins by its acyl-transferase activity during the differentiation process. The transglutaminase-catalyzed cross-link content of both the cytosolic and the nuclear protein fractions increased while NB4 cells underwent cellular maturation. Inhibition of cross-linking activity of TG2 by monodansylcadaverin in these cells led to diminished nitroblue tetrazolium (NBT) positivity, production of less superoxide anion, and decreased expression of $gp91^{phox}$, the membrane-associated subunit of NADPH oxidase. Neutrophils isolated from TG2-/- mice showed diminished NBT reduction capacity, reduced superoxide anion formation, and down-regulation of the $gp91^{phox}$ subunit of NADPH oxidase, compared with wild-type cells. These results clearly suggest that TG2 may modulate the expression of genes related to neutrophil functions and is involved in the process of differentiation of the neutrophil. To further investigate the role of TG2 in the differentiation process, RNA interference-mediated stable silencing of TG2 in NB4 cells (TG2-KD NB4) coupled with whole genome microarray analysis was performed. Our experiment revealed that TG2 contributes to the expression of a numerous ATRA-regulated genes. In the TG2-KD NB4 cells during ATRA-induced differentiation a large number of genes related to neutrophil granulocyte function remained partially suppressed. Down-regulation of these genes led to reduced adhesive, migratory and phagocytic capacities of neutrophils and less superoxide production. ATRA-controlled down-regulation of the genes, which are involved in cell cycle control and cell proliferation was less significant and was manifested in a higher proliferative rate of TG2-KD NB4 cells. Since we observed that the induction of CC-chemokines (CCL2, -3, -22, -24), which are responsible for the development of retinoic acid syndrome (RAS) in ATRA treated APL patients, were significantly lower in TG2-silenced NB4 cells, significance of TG2 may have far-reaching consequences in clinical aspects of ATRA treatment of APL patients.

Based on our results we demonstrate a complex regulatory effect of TG2 upon the retinoic acid-mediated differentiation of myeloid cells and propose that reduced expression of TG2 in differentiating APL cells may suppress effector functions of neutrophil granulocytes and therefore moderate the ATRA-induced hyper-inflammatory response in RAS.

PUBLICATIONS AND POSTERS

This thesis is built on the following publications:

Csomos K, Nemet I, Fesus L, Balajthy Z.: Tissue transglutaminase contributes to the all-trans retinoic acid induced differentiation syndrome phenotype in the NB4 model of acute promyelocytic leukemia. *Blood. (2010)*
IF: 10,55

Balajthy Z, **Csomos K**, Vamosi G, Szanto A, Lanotte M, Fesus L.: Tissue-transglutaminase contributes to neutrophil granulocyte differentiation and functions. *Blood.* **108**(6):2045-54. (2006)
IF: 10,37

Other publications:

Keresztessy Z, Csosz E, Harsfalvi J, **Csomos K**, Gray J, Lightowlers RN, Lakey JH, Balajthy Z, Fesus L.: Phage display selection of efficient glutamine-donor substrate peptides for transglutaminase 2. *Protein Sci.* **15**(11):2466-80 (2006)
IF: 3,46

Majai G, Sarang Z, **Csomos K**, Zahuczky G, Fesus L.: PPARgamma-dependent regulation of human macrophages in phagocytosis of apoptotic cells. *Eur J Immunol.* **37**(5):1343-54 (2007)
IF: 4,66

Poster presentations on international congresses:

Balajthy Z, **Csomos K**, Fesus L.: Retinoic acid-induced tissue-transglutaminase contributes to neutrophil granulocyte differentiation by modulating the expression of $gp91^{phox}$. *8th International Conference on Protein Crosslinking and Transglutaminases.* Lübeck, Germany (2005)

Majai Gy, Sarang Zs, **Csomos K**, Fesus L.: PPAR gamma dependent programming of macrophage capacity for phagocytosis of apoptotic cells. *13th ECDO Euroconference on Apoptosis.* Budapest, Hungary, (2005)

Csomos K, Balajthy Z, Zahuczky G, Fesus L.: Studying the role of tissue-transglutaminase in neutrophil granulocyte differentiation. *32nd FEBS Congress Molecular Machines.* Vienna, Austria (2007)

Csomos K, Balajthy Z, Zahuczky G, Fesus L.: Studying the role of tissue-transglutaminase in neutrophil granulocyte differentiation. *9th International Conference on Protein Crosslinking and Transglutaminases.* Marrakesh, Morroco (2007)

Csomos K, Nemet I, Fesus L, Balajthy Z.: Tissue transglutaminase contributes to all-trans retinoic acid induced gene expression remodelling in acute promyelocytic leukemia. Remodelling in Acute Promyelocytic Leukemia. *Gordon Research Conferences: Transglutaminases In Human Disease Processes.* Davidson College, NC, US. (2010)