INVESTIGATION OF GLUCOCORTICOID-INDUCED APOPTOSIS IN IMMATURE AND MATURE T-CELL SUBPPOPULATIONS

PhD Thesis

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1. Introduction

1.1 The effects of glucocorticoid hormones

Glucocorticoid hormones (GC) are produced by the cells of zona fasciculata in the adrenal gland. The most important GC in human is cortisol, in murine the corticosterone. The release of the hormones is strictly regulated by the hypothalamus-pituitary-adrenal axis, and can be induced by both psychic and physical stress. GCs have multiple physiological effects; they play role in the metabolism of carbohydrates, fats, proteins, influence development, water and electrolyte balance, vasculature and the immune system. Biologically active GCs are also produced by the thymus, intestines, brain and adipose tissue.

GCs have significant effect on the immune system. They influence the apoptosis of developing thymocytes, regulate the signal transduction through the T cell receptor (TcR). They are also important in the function of mature T cells; they are key in the maintenance of a proper T cell immune response. The synthetic analogues (e.g. dexamethasone) are commonly used in the clinical practice in the treatment of autoimmune diseases, haematological malignancies and allergies, because they induce the apoptosis of leukemic cell, developing thymocytes and certain mature, activated T cells.

1.2 The glucocorticoid receptor

GCs exert their effect through the GC receptor (GR). GR is a member of the nuclear receptor superfamily, a ligand-dependent transcription factor. It is a conserved protein; the amino acid sequence of the human and murine GR is 90% the same. There are two isoforms of the human GR, which are the mostly studied, the GRα and GRβ. GRα is localised in the cytoplasm, this is the dominant form, and beta is constantly found in the nucleus. The function of GRβ is not well-known. The presence of GRα and GRβ was also proved in mouse. GRα is the more important isoform, and the function of GRβ is similar to the human GRβ. GR is expressed in most cells and tissues.

1.3 Signal transduction through the glucocorticoid receptor

GCs exert their effects mostly through two pathways. These pathways are the slowly developing genomic and the faster non-genomic pathways.

1.3.1 Genomic effects

The genomic effects were described first. GR is inactive in the cytoplasm and bound to protein-complex (e.g. heat shock proteins- (Hsp-) 90, Hsp-70, p23). GR dislocates from the protein-complex upon ligand binding and dimerise, then translocates to the nucleus, where it binds to certain parts of the DNA, called GRE (glucocorticoid responsive elements) and influences the gene expression. It takes long time, hours or days, to develop its gene expression changing effects. The activated GR as a transcription factor can influence the gene expression of several genes directly or indirectly by binding to other transcription factors (e.g. AP-1, NF-κB, NFAT). It can induce (e.g. annexin-1, interleukin- (IL-) 10) or inhibit (e.g. IL-2, Il-1β) the expression of certain genes.

1.3.2 Non-genomic effects

The non-genomic effects, which can develop within minutes are not mediated by transcriptional changes, but develop through other alternative pathways. The following non-genomic pathways have been described so far:

1. GCs are able to influence membrane-transport processes in certain cell types through their direct membrane effect. High-dose steroid treatment changes the mobility of membrane lipids, because the GCs able to influence the physical-chemical characteristics of the plasma membrane due to their lipophilicity.
2. The membrane GR (mGR) was observed on monocytes and B cells. In patients with rheumatoid arthritis and ankylosing spondylitis the expression of mGR is increased on B cells and monocytes, and might have pathological effect in these diseases.
3. GR is able to bind to cytoplasmic proteins and this way influence signal transduction pathways. In human $CD4^+$ T cells after TcR stimulation the GR associated with the TcR signalling molecules, Lck, Fyn. Short-term DX treatment inhibited the Lck/Fyn phosphorylation after TcR activation, which was the result of the dissociation of the GR-TcR-Lck-Fyn multi-molecular complex. In Jurkat, human T cell line, high dose, short term DX treatment caused temporary ZAP-70 phosphorylation without TcR activation. This activation could be inhibited by RU486. In Jurkat cells the GR associated with the ZAP-70 kinase, and the GR and ZAP-70 formed multi-molecular complex with Hsp-90. The GCs are able to effectively inhibit T cells functions. The above mentioned processes may take part in this inhibition.
4. After ligand binding the activated GR can translocate to the mitochondria in lymphoid and non-lymphoid cells, where it might contribute to the initiation of the apoptotic cascade. In the previous work of or research group we have observed in double positive (DP) thymocytes that after 30 min GC-analogue treatment the GR rather translocated to the mitochondria than to the nucleus, and the subsequent decrease of the mitochondrial membrane potential indicated the activation of apoptosis in this cells. This translocation correlated with the high GC-induced apoptosis sensitivity of the DP cells. We do not know exactly how the mitochondrial translocation of the GR contributes to the apoptosis of sensitive cells.

1.4 Apoptosis

Apoptosis, or programmed cells death, plays part in several physiological processes, including the development of immune cells and immune response. There are several forms of cell death, all of them involves many, complex molecular processes.

Galluzzi et al., in their newest, 2018 recommendation for Nomenclature Committee on Cell Death, distinguished two forms of cell death, the accidental cell death (ACD) and regulated cell death (RCD). ACD can be caused by extreme physical, chemical and mechanical stresses. RCD is a strictly regulated complex process. Apoptosis belongs to RCD. There are three main apoptotic pathway (extrinsic, intrinsic and caspase-independent), which can be divided into three phases; initiation (activation), execution and cell death.

1.4.1 Extrinsic apoptosis

Extrinsic apoptosis is initiated by extracellular stress signals, which are recognized by cell surface receptors. These receptors are the death receptors (DR). The mostly studied DRs are tumour necrosis factor (TNF) receptor, (TNFR), Fas receptor (FasR), TNF-related apotosis-inducing ligand (TRAIL). Their intracellular domain, the death domain, provide binding site for intracellular proteins after the activation of receptors. The complex is recognized by the death-inducing signalling complex (DISC), which then activates the

procaspase-8. In type I cells (e.g. mature lymphocytes) the large amount of activated caspase-8 activates caspase-3 and -7 and this is enough to the apoptosis of the cell. In type II cells (e.g. hepatocytes, pancreatic β cells, majority of cancer cells) low amount of caspase-8 gets activated and caspase-3 and -7 are inhibited by XIAP (X-linked inhibitor of apoptosis protein). Caspase-8 has to cleave Bid, and the truncated Bid then migrates to the mitochondria and activates the intrinsic apoptotic pathway with the BH3-only proteins.

1.4.2 Intrinsic apoptosis

The intrinsic apoptotic pathway can be activated by irreversible DNA damage, high concentration of intracellular Ca^{2+}, oxidative stress, growth factor withdrawal, stress of the endoplasmic reticulum. It is controlled by the Bcl-2 family proteins. The common characteristic of these proteins that they have minimum one, maximum four conserved Bcl-2 homology (BH) domains. The family can be divided into pro-apoptotic (Bax, Bak, Bad, Bcl-x_S, Bid, Bik, Bim, Hrk) and anti-apoptotic proteins (Bcl-2, Bcl-x_L, Bcl-W, Bfl-1, Mcl-1) based on whether they activate or inhibit apoptosis. The pro-apoptotic proteins have two subgroups the Bax, or multi-domain pro-apoptotic proteins (Bax, Bak, Bok, Mtd), which have BH1-BH3 or BH1-BH4 domains and the BH3-only group (Bim, Bid, Bad, PUMA, Noxa) which has only BH3 domain. The BH3-only proteins are able to directly activate the pore-forming multi-domain pro-apoptotic proteins, or they can inhibit the anti-apoptotic proteins.

The activated Bax and Bak form a molecular complex and pore on the outer membrane of the mitochondria (MOMP; mitochondrial outer-membrane permeabilisation) and this way they contribute to the release of cytochrome c from the inner membrane of the mitochondria. The anti-apoptotic proteins, like Bcl-2, Bcl-x_L, try to inhibit this process by neutralizing the pro-apoptotic proteins and inhibiting the release of cytochrome c to the cytoplasm. The formation of the pore on the mitochondrial outer membrane ("point of no return") inhibits ATP synthesis, toxic proteins are released to the cytoplasm such as cytochrome c, apoptosis inducing factor (AIF), endonuclease G (EndoG), Smac (or Diablo) and HtrA2 (or Omi), blocks the respiratory chain and leads to the accumulations of reactive oxygen species. The cytochrome c in the cytoplasm associates with Apaf-1 (apoptosis protease-activating factor-1) and pro-caspase-9, causing the activation of the latter. This multi-molecular complex is the apoptosome. The caspase-9 then activates caspase-3 and -7 which leads to the death of the cell.

1.5 The effect of glucocorticoids on the apoptosis of thymocytes and T cells

The effects of GC on the immune system have been well known. Injection of high-dose GC causes rapid involution of the thymus because of the depletion of thymocytes and TEC. Interestingly the thymocytes and TEC are able to produce GCs. But GC synthesis has not been described in SP thymocytes and peripheral naïve or activated T cells. It seems that the GC synthesis is a unique characteristic of the developing thymocytes.

GCs are also able to protect cells from the apoptosis induced through TcR in certain conditions. In lower concentrations the GCs can inhibit the signal transduction through the TcR in thymocytes, when they receive signals through the two receptors at the same time. This can protect those thymocytes from apoptosis, induced by the TcR, which would be killed during the negative selection. This is the so called mutual antagonism model. In the

thymocytes, which receive the two signals at the same time, the Bcl-2 gets upregulated. Other research group, based on the mutual antagonism model, has proved that GCs play role in the positive selection as well.

Other research groups have described the cross-talk between the GR and TcR signalling molecules in naïve T cells; GCs inhibited the Lck and Fyn molecules. It is supposed that GCs, produced by the adrenal gland, primarily influence the systemic immune responses, while the hormones, produced locally in the thymus, contribute to the development of the T cells.

The DP cells are the most sensitive to the GC-induced apoptosis among the thymocyte subpopulations, despite the fact that these cells express the lowest level of GR. We have observed the translocation of the GR to the mitochondria and the decrease of the mitochondrial membrane potential in these cells upon DX treatment. In a TcR transgenic mouse model we have observed that those thymocytes, which survive the T cell selection, upregulate the anti-apoptotic Bcl-2 molecule.

Bcl-2 proteins play an important role in the DX-induced apoptosis of thymocytes. It has been described that Bax and Bak are important in the DX-induced apoptosis. GC influences the expression level of Bcl-2 proteins, due to their genomic effects, they change the expression level of Bim and Bcl-x_L. These results together with ours strengthen the possibility of the importance of intrinsic apoptotic pathway in the GC-induced apoptosis of DP thymocytes.

We have not examined the effects of GCs only on DP thymocytes. In our other work we have observed the alterations in the cellular composition of lymphoid organs after *in vivo* administration of DX. We focused on the regulatory T cells (Tregs) which are key in the control of the immune system. We have found that the thymic Tregs (tTregs) showed certain resistance to the DX-induced apoptosis, in contrast with that the splenic Tregs (pTregs) were sensitive to the DX-induced apoptosis. But the molecular background of this phenomenon is still not fully understood.

To summarize, the mitochondrial translocation of the GR, and this way its non-genomic effect, presumably play an important part in the apoptosis of thymocytes, however the exact mechanism is not fully understood. Nevertheless, the different reaction of other cells to the *in vivo* administered DX raises the possibility that the GR activates other apoptotic pathways probably through other non-genomic processes in these cells.

2. Aims

GCs play an important role in the development and apoptosis of immune cells, but the different cells react with different sensitivity to the steroid treatment. Among the thymocyte subpopulations the DP cells are the most sensitive to the DX-induced apoptosis. This is presumably caused by one of the non-genomic effects of the GR, the translocation of the activated GR to the mitochondria, and the consequential decrease of the mitochondrial membrane potential. But the exact molecular mechanism of the process is not completely understood.

In our work we wanted to analyse the effect of GCs on the apoptotic processes of DP thymocytes, especially the interaction between the GR and the Bcl-2 proteins, and the activation of caspases.

1. Analysis of the interaction between the GR and Bcl-2 proteins in DP thymocytes
2. Observing the effect of short term DX treatment on the subcellular distribution of Bcl-2 proteins
3. Detection of the presence of cytochrome c in the cytoplasm
4. Description of the kinetics of caspases' activation

Previously we have observed that the different T cell subgroups showed different sensitivity to the i*n vivo* DX treatment, the tTregs were especially resistant compared to the other cells.

The processes that are responsible for this are not well known, therefore we wanted to investigate them in more detail.

1. Investigation of early apoptotic signs with Annexin V in $CD4^+$ thymocytes, splenocytes, tTregs and pTregs
2. Description of the kinetics of caspases' activation in $CD4^+$ thymocytes and splenocytes and Tregs
3. Analysis of co-localisation between GR and Foxp3 in tTregs and pTregs

3. Materials and methods

3.1 Mice

Four to six weeks old *BALB/c* mice were used to all experiments. The animals were kept under conventional conditions. They were provided with pelleted rodent chow and water *ad libitum*. All animal experiments were carried out in accordance with the regulations of Animal Welfare Committee of University of Pécs (#BA 02/2000-16/2015).

3.2 *In vitro* GC-analogue treatment of isolated thymocytes and splenocytes

Mice were sacrificed, and then thymi and spleens were removed. The organs were mechanically homogenized, then filtered. $5x10^7$ thymocytes and splenocytes were treated with 10^{-6}M DX in serum-free RPMI-1640 medium. Control samples were kept under the same conditions for the same time in the presence of the solvent.

3.3 Antibodies

For flow cytometry the following antibodies were used: anti-CD4-PE-Cy5, anti-CD4-PE, anti-CD8-PE, anti-CD8-PECy5, anti-CD25-APC. For the analysis of activated (cleaved) caspases rabbit anti-caspase-3, rabbit anti-caspase-8 and rabbit anti-caspase-9 were used. As secondary antibody goat anti-rabbit IgG-Alexa Fluor 488 was used.

For confocal microscopy the following Abs were used anti-CD4-Pacific Blue, anti-CD8-Pacific Orange, anti-GR-FITC, anti-FoxP3-Alexa Fluor 647 and rabbit anti-Bak, -Bax, -

Bcl-xL and anti-Bim with goat anti-rabbit IgG-Cy3 secondary Ab and goat anti-rabbit IgG-FITC secondary Ab.

For western blot analysis of the activated caspases the following antibodies were used: rabbit anti-caspase-3, -8, -9, with anti-rabbit IgG-peroxidase as secondary antibody. Mouse anti-cytochrome c with anti-mouse-PO as secondary antibody, rabbit anti-Bax with anti-rabbit-PO were used to detect apoptotic proteins. For reprobing mouse anti-β-actin and anti-cytochrome c were used, both with anti-mouse-PO as secondary antibody.

Anti-GR was used for immunoprecipitation. For western blot analysis of the immunoprecipitated samples anti-Bak, anti-Bax, mouse anti-Bcl-xL with anti-mouse-PO as secondary Ab, and rabbit anti-Bim with anti-rabbit-PO as secondary Ab. For reprobing anti-GR with anti-mouse-PO as secondary Ab.

3.4 Annexin V labelling

To detect the early stage of apoptosis in thymocytes and splenocytes Annexin V staining was performed. After cell surface labelling of 10^6 cells with anti-CD4-PE, anti-CD8-PECy5, anti-CD25-APC, Annexin V- FITC labelling was performed. Samples were analysed with flow cytometer.

3.5 Mitotracker chloromethyl-X-rosamine (CMX-Ros) labelling for confocal microscopy

CMX-Ros is a fluorescent dye that stains mitochondria in cells. 1^{06} thymocytes and splenocytes were incubated in serum-free RPMI-1640 containing 10 µl CMX-Ros stock solution for 30 min at 37°C. For confocal microscopy cell surface labelling of thymocytes with anti-CD4-Pacific Blue, anti-CD8-Pacific Orange and intracellular labelling with rabbit anti-Bax primary and anti-rabbit IgG-FITC secondary antibody was performed.

3.6 Labelling for confocal microscopy

After 30 minutes DX treatment anti-CD4-Pacific Blue and anti-CD8-Pacific Orange labelling of thymocytes were performed in binding buffer, then cells were fixed. The intracellular labelling was performed in saponin buffer with rabbit anti-Bak, -Bax, -Bcl-xL and -Bim as primary antibodies and anti-rabbit IgG-Cy3 as secondary antibody, then with 1 µg/ml anti-GR-FITC antibody.

3.7 Analysis of GR-Foxp3 interaction with confocal microscopy

The negatively separated CD4 positive thymocytes and splenocytes (EasySep Mouse $CD4^+$ T Cell Isolation Kit) were further separated positively for CD25 (EasySep Mouse CD25 Regulatory T Cell Positive Selection Kit). After 30 min of DX treatment the cell were labelled with anti-CD4-Pacific Blue, anti-GR-FITC and anti-Foxp3-Alexa Fluor 647 with the Foxp3 Transcription Factor Staining Buffer Set to analyse the GR-Foxp3 interaction.

3.8 Confocal microscopy

Visualization and analysis of samples were carried out using an Olympus Fluoview 300 confocal microscope with an Olympus Fluoview FV1000S-IX81 image acquisition software system. Sequential scanning was used for image acquisition. Signals were collected from cells in 3-3 frames and Bak, Bax, Bcl-xL, Bim-GR, Bax-CMX-Ros and GR-Foxp3 morphological association was analysed with ImageJ software using co-localization plug-in. Then co-localized pixels between Cy3-GR, CMX-Ros-Bax and GR-Foxp3 were counted. One hundred cells per sample were analysed altogether this approach.

3.9 Labelling of activated caspases for flow cytometry

10^6 thymocytes and splenocytes were treated with DX for 0,5, 1, 2, 3 and 6 hours. The cells were labelled with anti-CD4-PE-Cy5, anti-CD8-PE and anti-CD25-APC. The intracellular labelling was performed with caspase-3, -8, -9 and anti-rabbit-IgG-Alexa Fluor 488. The samples were analysed by flow cytometer.

Flow cytometry

Samples were measured and analysed in, using the CellQuest Pro software. Thymocyte and splenocytes subpopulations were analysed separately based on their cell surface CD8, CD4 and CD4+CD25 expression, for Alexa Fluor 488 intensity detected in FL1 channel and for CMX-Ros intensity detected in FL2 channel. Fluorescent histogram plots were used to compare the ration of active caspase-3, -8, -9 expressing cells (Alexa Fluor 488 positive) or the median fluorescent intensity of CMX-Ros of different samples.

3.11 Subcellular fractioning

Mitochondrial Isolation Kit was used to separate cytoplasmic, mitochondrial and nuclear fractions from thymocytes, according to the manufacturer's instructions with minor modifications. The mitochondrial and the cytoplasmic fractions were either used for immunoprecipitation and then for western blot. To use the mitochondria for immunoprecipitation the mitochondrial pellet was further lysed in TEGM lysis buffer for 30 minutes and then centrifuged at 13 000 rpm for 10 minutes and the supernatant was used for immunoprecipitation.

3.12 Immunoprecipitation

The cytoplasmic and mitochondrial fractions were incubated with the precipitating antibodies in blocking buffer under continuous rotation overnight. Then Protein-G was added to the samples, and they were incubated for another 2 hours under continuous rotation. Finally, the samples were washed five times in PBs, then the immune complexes were removed from the Protein-G with boiling for 3 min in SDS sample buffer

3.13 Western blot

Mitochondrial and cytosolic fractions, after they were boiled in SDS sample buffer, were loaded to SDS polyacrylamide gel electrophoresis. The primary antibodies; anti-caspase-3, -8, -9 and anti-Bax and in the case of the immonoprecipitated samples: anti-Bak, -Bax, -Bcl-xL, -Bim and anti-GR. Blots were reprobed with anti-β-actin and anti-cytochrome c antibodies. The visualization of the blots was performed by enhanced chemiluminescence according to the manufacturer's instruction. Luminescent light signals were detected with Fujifilm LAS 4000 blot documentary system.

3.14 Analysis of blots

The blots were analysed with densitometry performed with the Image J software. Relative densities of proteins were normalized to the densities of β-actin, cytochrome c and GR.

3.15 Statistical analysis

GraphPad Prism program was used to create artwork and perform the statistical analysis using Student's t-test. $p < 0.05$ was considered statistically significant. Data are presented as mean ± SEM.

4. Results

4.1 DX induced changes in the co-localisation between GR and members of Bcl-2 protein family

We set out to find potential molecular partners for the activated GR in the mitochondrial apoptotic pathway. To this end we investigated possible protein interactions between the GR and Bcl-2 family proteins, which are responsible for the control of the mitochondrial membrane potential. The co-localization of GR and Bak, Bax, Bcl-x_L or Bim was analysed in DP thymocytes before and after 30 minutes of high dose DX treatment. We found that the GR co-localized to some extent with all four investigated Bcl-2 family proteins. To quantify the rate of co-localization, we calculated and compared the number of co-localized pixels in individual DP cells after 30 minutes of DX treatment to their controls. After DX treatment the co-localized pixel number minimally changed between Bak and GR but decreased slightly between Bax and GR. The co-localization between GR and Bcl-x_L decreased significantly after DX treatment. We observed a remarkable, but statistically not significant, increase in the co-localization of Bim and GR upon 30 min DX treatment.

4.2 The GR interacts with members of the Bcl-2 protein family in the cytoplasm and the mitochondria of the thymocytes

To confirm our confocal microscopic results, we investigated the interaction of the GR with Bcl-2 family member proteins: Bak, Bax, Bcl-x_L and Bim proteins in thymocytes using co-immunoprecipitation with anti-GR antibody. We also wanted to elucidate whether the high dose DX treatment changed the active GR–Bcl-2 family protein complexes' subcellular distribution. Therefore, we performed subcellular fractionation and isolated cytoplasmic and mitochondrial fractions from 30 minutes DX or vehicle-treated, unseparated thymocytes. After subcellular fractionation immunoprecipitation was performed with anti-GR antibody and then the samples were further analysed by western blot to visualize the co-precipitated Bcl-2 family proteins.

Bak co-precipitated with the GR, and upon DX treatment the Bak–GR co-precipitation increased in the cytoplasmic and slightly changed in the mitochondrial fraction. We also observed the co-precipitation of Bim with the GR. Bcl-x_L also co-precipitated with GR. The rate of their co-precipitation increased in the cytoplasmic and decreased in the mitochondrial fraction upon DX treatment in comparison to the control. Finally, the GR–Bim association changed only minimally in the cytoplasmic fraction, but remarkably increased in the mitochondrial compartment. This pronounced mitochondrial accumulation of Bim suggests its potential role in the mitochondrial (intrinsic) apoptotic pathway in the GC-induced thymocyte apoptosis.

4.3 DX treatment-induced mitochondrial accumulation of Bax

In the case of Bax we could not confirm the co-localization, observed by confocal microscopy, with co-immunoprecipitation (data not shown). Therefore, we investigated whether the high dose DX treatment caused any redistribution of Bax between the cytoplasmic and mitochondrial fractions of thymocytes, and we have found that Bax accumulated in the mitochondrial fraction after 30 min of DX treatment, compared to the control. This result was confirmed by confocal microscopy; the number of Bax–CMX-Ros co-localized pixel number increased upon 30 minutes of DX treatment.

4.4 Kinetics of caspases' activation in DP thymocytes

We investigated the activation of caspases in DP thymocytes. The ratio of DP cells containing cleaved caspase-9 increased significantly after 2 and 3 hours of DX treatment. The percentage of DP cells in which active caspase-3 was detected showed increase already after 1 hour of DX treatment, and after 2 and 3 hours of DX treatment the rate of DP cells having active caspase-3 increased significantly. The activation of caspase-9 together with the cleavage of caspase-3 implied the activation of the intrinsic, mitochondrial apoptotic pathway upon DX treatment. The ratio of active caspase-8 containing DP cells was slightly elevated upon 0.5 and 1 hour of DX treatment, and this increase continued and became significant after 2 and 3 hours DX treatment showing a similar tendency to the active caspase-9. The changes in caspase-9 activation seemed to be more pronounced than in the case of caspase-8 after 2 and 3 hours of DX treatment, which may suggest a pivotal role of caspase-9 in DX-induced thymocyte apoptosis.

4.5 DX-induced caspase activation and cytochrome c release to the cytoplasm in thymocytes

In vitro DX-treated thymocytes were compared for active caspase-3, -8, -9 and cytochrome c levels. One hour, high dose DX treatment caused the significant increase of cytochrome c level in the cytoplasm. We observed the significant elevation of active caspase-9, -3 levels compared to the control after 3 hours of high dose DX treatment which are characteristic signs of the activation of the intrinsic (mitochondrial) apoptotic pathway.

4.6 Analysis of Annexin V in $CD4^+$ T cells and Tregs

After cell surface labelling with CD4, CD8 and CD25 Annexin V labelling was performed on thymocytes and splenocytes before and after 0, 2 and 4 hours of DX treatment. In thymic $CD4^+$ T cells after 4 h of DX treatment the ratio of Annexin V positive cells was significantly increased. In tTregs we have not observed the apoptosis-inducing effect of DX after 4 hours. In splenic $CD4^+$ T cells the ratio of Annexin V positive cells was already increased after 2 hours and this increase continued till 4 hours, but to a lesser extent. In pTregs we have observed increase at two hours, after 4 hours no difference was found. Interestingly the ratio of Annexin V positive cells also increases in the control $CD4^+$ thymocytes and tTregs. But this phenomenon was not observed in untreated $CD4^+$ splenocytes and pTregs.

4.7 Analysis of caspases' activation in $CD4^+$ T cells and Tregs

We have performed the labelling of active caspases in thymocytes and splenocytes as well. The cell surface was labelled with CD4, CD8 and CD25 to distinguish the populations of interest. Both in the case of thymus and spleen we have observed that the ratio of cells containing activated caspases was much lower than in the case of DP thymocytes.

After DX treatment the ratio of active caspases containing thymic $CD4^+$ T cells was significant, and this tendency continued further and significant increase has been observed at 6 hours. In the case of tTregs we have only observed minimal increase upon DX treatment. Only the ratio of caspase-3 containing cells was significant after 6 hours.

The ratio of caspase positive splenic $CD4^+$ T cells was significantly higher after 2 and 3 hours of DX treatment compared to controls and this tendency remained similar after 6 hours, but the increase was not as pronounced as in the case of thymic $CD4^+$ T cells. To sum

up the ratio of caspase containing splenic cells was lower at every examined time point in splenic $CD4^+$ T cells than in the case of thymic $CD4^+$ T cells. The significant activation of caspase-3 and -8 was only observed after 3 hours of DX treatment in pTregs, which showed further increase after 6 hours of treatment, but this increase was lower than in the case of splenic $CD4^+$ T cells.

4.8 Investigation of GR and Foxp3 interaction with confocal microscopy

We have examined the possible connection between GR and Foxp3, a key transcription factor in Tregs, and we have also analysed whether this connection changes upon DX treatment. After 30 min of DX treatment we have investigated the co-localisation between GR and Foxp3 with confocal microscopy. GR showed co-localisation with Foxp3 without DX treatment both in thymic and splenic Tregs. This co-localisation further increased in pTregs upon DX treatment, but did not changed in tTregs.

5. Discussion

Glucocorticoid receptor signalling plays an important regulatory role in the selection and apoptosis of thymocytes. Besides the nuclear-, mitochondrial translocation of the ligand-bound GR might dictate GC-induced apoptosis sensitivity of the cells. In a previous study, we followed the ligand-induced GR trafficking in GC-sensitive $CD4^+CD8^+$ DP thymocytes upon short term in vitro GC treatment and demonstrated the GR translocation into the mitochondria, which well correlated with their pronounced GC-induced apoptosis sensitivity. However, the molecular events following the short-term GC treatment-induced mitochondrial translocation remained to be elucidated. In our present work we clarified that the GR regulates the mitochondrial apoptotic pathway of thymocytes in close collaboration with the Bcl-2 family proteins.

We observed both co-localization and direct molecular association of Bak with GR. After DX treatment this association was unchanged in the mitochondrial fraction but increased in the cytoplasm of thymocytes upon high-dose short-term DX treatment. Upon apoptotic stimuli, Bax translocates to the mitochondria where it forms a complex with Bak leading to mitochondrial pore formation. Our findings suggest that Bax has a primary role in the early phase of DX-induced apoptosis of thymocytes, although not associating directly with the GR. We cannot rule out the possibility that Bak also plays a role in GC-induced apoptosis, but probably joins at a later stage than we examined in our work. This is supported by earlier observations in thymocytes form Bax/Bak double KO mice which were completely resistant to GC-induced apoptosis, whereas Bax or Bak single KO mice thymocytes were still sensitive to GCs. These studies, with knock-out mice, have strengthened the importance of Bak in GC-induced apoptosis, but also have suggested that Bax and Bak may compensate for each other.

Our results showed the association of Bim, a BH3-only protein, with GR and their interaction increased especially in the mitochondrial fraction upon DX treatment. Supposedly Bim activates pro-apoptotic Bcl-2 proteins or inhibits anti-apoptotic ones and this way it induces the mitochondrial apoptotic pathway. Bim-/- knock-out mice showed impaired GC-induced apoptosis showing its important but not exclusive participation in this death process.

This is also supported by the results of other research groups. GCs have been found to induce the expression of Bim in murine thymocytes after 2 or 3 hours of DX treatment. Increased expression of Bim has correlated with increased sensitivity to GC-induced apoptosis.

GCs are widely used in the in the chemotherapeutic protocol of lymphoid malignancies, especially in paediatric acute lymphoblastic leukaemia. The better outcome of the diseases correlated with the ability of GCs to induce the transcription of Bim in certain patients. The increased expression of Bim correlated with GC-induced apoptosis sensitivity. Dysregulation of its gene expression has been found in solid and hematopoietic malignancies, where reduced expression correlated with increased disease risk, and single nucleotide polymorphisms have been associated with impaired responsiveness to anticancer therapies. Our results also support that Bim plays a crucial role in the initiation of GC-induced apoptosis of DP thymocytes; the increased association of Bim with the GR in the mitochondria may promote the activation and oligomerisation of Bax in the mitochondrial outer membrane.

Interestingly, we also observed interaction between Bcl-x_L, an anti-apoptotic member of the Bcl-2 family, and the GR during the DX-induced apoptotic processes. Bcl-x_L has been shown to retrotranslocate Bax from the mitochondria to the cytoplasm by binding to it and thus inhibiting its pro-apoptotic activity. We hypothesize that the interaction between the GR and Bcl-x_L would cause the inhibition of this particular Bcl-x_L function. After 30 minutes DX treatment the GR bound ratio of Bcl-x_L increased in the cytoplasmic but decreased in the mitochondrial fraction which suggests that Bcl-x_L, after translocating to the cytoplasm from the mitochondria, binds to the GR, and this sequestration could abolish its antagonistic effect on the apoptotic process. This hypothesis about the inhibitory effect of the GR on Bcl-x_L is supported by the result of another research group where it has been observed that the expression of Bcl-x_L decreased significantly after 2 or 3 hours of DX treatment. Consequently, the inhibitory effect of DX on Bcl-x_L can be observed both on protein and gene expression level.

However, the co-localization between Bcl-x_L and the GR decreased significantly after DX treatment, which might be due to the fact that the co-localization results are only from DP cells and it gives the overall ratio of co-localization, both in the cytoplasm and the mitochondria, while unseparated thymocytes were used for the co-immunoprecipitation experiment and the cytoplasmic and mitochondrial fractions were analysed separately.

The translocation of Bax between the cytoplasm and the mitochondria is an important regulator of the intrinsic apoptotic pathway. In our results the rate of co-localization between Bax and the GR slightly changed upon DX treatment, but we could not confirm the co-localization, observed by confocal microscopy, with co-immunoprecipitation experiments. Co-localization expresses molecular proximity, but does not reflect necessarily direct molecular interaction between two molecules. In the case of Bax, where the co-localization with the GR was not confirmed by co-immunoprecipitation, the results suggest that the two molecules were very close to each other, but there was no direct interaction between them. According to our results GR, a 94 kDa molecule, associates with other members of the Bcl-2 protein family, which are in the vicinity of Bax. It is known from the work of others that these Bcl-2 proteins interact with each other, which may explain the proximity of the GR to Bax without direct association. Besides we detected a clear redistribution of Bax from the cytoplasm to the mitochondria which correlated with the results of others suggesting the

central role of Bax in DX-induced apoptosis of thymocytes. Bax trafficking between the mitochondrial outer membrane and the cytoplasm is a key regulator of the intrinsic (mitochondrial) pathway of apoptosis. Bax oligomerisation in the mitochondrial membrane leads to the formation of a permeability pore, which causes the decrease of the mitochondrial membrane potential, as it has been detected in our previous experiments.

Caspases are important effectors of both, intrinsic and extrinsic, apoptotic pathways. In our experiments we analysed the kinetics of caspases' activation from 0.5 to 3 hours of DX treatment. We observed significantly increased number of DP thymocytes containing active, cleaved caspase-3, -8, -9 after 2 and 3 hours of DX treatment. After 1 hour of DX treatment, the caspase-3 activation was probably the result of caspase-9 activation following the decrease of the mitochondrial membrane potential observed after 30 minutes DX treatment in our previous work. But the activation of caspase-3 after 1 hour of DX treatment may be partially the result of the activation of parallel apoptotic pathways. These include ceramide and sphingosine generation which were reported to be able to induce caspase-3 activation in a mitochondria independent manner. The prominent caspase-9 activation after 2 hours DX treatment was followed by remarkable caspase-3 activation after 3 hours DX treatment. The number of DP cells containing activated caspase-9 was almost doubled after 2 hours and the number of cleaved caspase-8 containing DP cells increased significantly but to a lesser extent than caspase-9. This observation suggests that the activation of caspase-9 may be prior to caspase-8 activation and strengthen the importance of the mitochondrial apoptotic pathway in DX-induced apoptosis of DP thymocytes.

Our results are supported by the work of other research groups. Several knock-out models have been generated already, where one or more members of the Bcl-2 family or caspases were inactivated and thus, the deficiency of these proteins can be studied effectively. These models have provided an important insight into the different apoptotic pathways. For example, caspase-9-/- KO thymocytes have been found to be resistant to DX-induced apoptosis, but remained sensitive to apoptosis induced by TNF-α, α-CD95. Apaf-/- KO thymocytes have shown only partial resistance to DX-induced apoptosis and impaired procaspase-8 processing, but were sensitive to apoptosis induced by Fas ligation. GC-induced thymocyte apoptosis has been unaffected in Bid-deficient mice suggesting the dispensable role of the extrinsic apoptotic pathway in GC mediated cell death. On the other hand, using small peptide inhibitors of caspases have shown the importance of caspase-3 and -8 in GC-induced thymocyte apoptosis, but the specificity of these inhibitory molecules might be unclear. Some results have suggested the primary role of caspase-9 in GC-induced apoptosis. However, others have not supported these findings. The activation of caspase-8 could also be the result of the activation of caspase-9 either through the release of cathepsin B from lysosomes leading to caspase-8 activation or through the activation of caspase-3 and -6, which then cleaves caspase-8. But the activation of caspase-8 can be the result of the induction of other apoptotic pathways activated by GCs including; ceramide and sphingosine production, Cyclin-dependent kinase 2 activation, or as already mentioned above, the lysosomal release of cathepsin B.

In our works, where we have analysed the effect of *in vivo* administrated DX on the cellular composition of lymphoid organs, and we have found that the ratio of Tregs in the thymus significantly increased upon DX treatment. This increase was observed due to the apoptosis of GC-sensitive DP thymocytes and CD4 and CD8 SP thymocytes. This suggested that tTregs might have resistance to GC-induced apoptosis. We have not observed the increase of Tregs in the spleen. The total cell count of the spleen significantly decreased upon DX treatment and pTregs just like $CD4^+$ T cells died. We have concluded that the GC sensitivity of pTregs is different form the tTregs'.

The thymic $CD4^+$ T cells contained significantly more activated caspase-3, -8, -9 after 2 hours of DX treatment and after 4 hours the ratio of Annexin V positive cells was significantly higher. But in contrast with this in the tTregs we have only seen minimal caspase activation, and we have not found any difference after Annexin V labelling. This suggest that tTregs are less sensitive to the DX-induced apoptosis than the $CD4^+$ thymocytes and DP cells. In the case of the spleen the ratio of active caspases containing $CD4^+$ T cells was significantly higher after 2 and 3 hours of DX treatment, and after 3 hours the ratio of activated caspase-3 and -8 positive cells was also higher, and the Annexin V positivity of both cell populations was increased after 2 hours of treatment. This result gives the impression that the apoptotic sensitivity of Tregs in more expressed than that in the tTregs and more similar to the splenic $CD4^+$ T cells.

Only a few articles handle with the effect of GCs on the apoptosis of Tregs, especially with the activated apoptotic pathways. And most of these works analysed the effect of GCs on the number of Tregs in animal model of certain illnesses, and the results are sometimes inconsistent. In murine it has been described that DX caused the increase of Tregs in peripheral lymphoid organs and blood. In contrast with that in asthma and multiple sclerosis animal models GCs caused the decrease of the Tregs. The human results are also contradictory: asthma patients treated with steroid showed higher ratio of circulating Tregs, and similar phenomenon was observed in autoimmune patients; in patients with systemic lupus erythematosus, who received steroid therapy, had higher number of $CD25^+$ T cells, than in those who had no steroid, or were healthy. Other research groups observed the opposite effect of GCs. In Tregs, isolated from human peripheral blood, it was found that these cells are more sensitive to the DX induced apoptosis, than the effector T cells. But this sensitivity was compensated with minimal dose of IL-2. It makes difficult to compare the results because the experiments were made with different dose of DX and the markers used for the characterisation of Tregs were also different.

We have also analysed the interaction of GR and Foxp3. We have observed the co-localisation of the molecules in untreated cells, and the DX treatment caused increase in the co-localisation in pTregs, but had no effect on tTregs.

In conclusion, our results demonstrate the complexity of early steps of the DX-induced mitochondrial apoptotic pathway in GC sensitive, DP thymocytes. In the absence of its ligand some association could be observed between the GR and members of the Bcl-2 family (Bak, Bim, Bcl-xL) proteins. There is a constant turnover of the pro-apoptotic Bax between the mitochondrial outer membrane and the cytoplasm. When no apoptotic stimuli are present Bcl-xL retrotranslocates Bax from the mitochondrial outer membrane, thus the majority of Bax is located in the cytoplasm in an inactive conformation. Upon high dose GC treatment, the

liganded GR changes the equilibrium between the Bcl-2 family proteins, in such a way, which promotes apoptosis. GR translocates to the mitochondria where its interaction increases especially with Bim. Bim presumably activates Bax leading to the accumulation and permeability pore formation of Bax in the mitochondrial outer membrane, causing the decrease of the mitochondrial membrane potential, the release of cytochrome c and the activation of caspase-9. The pore formation of Bax in the mitochondrial outer membrane might be supported by the increased cytoplasmic association of the activated GR with Bcl-x_L, which interferes with the latter's inhibitory effect on the mitochondrial pore formation by Bax. The role of the GR–Bak association needs further investigations. Caspase-8 activation (extrinsic pathway) may be the result of the interaction of GR and other apoptotic pathways. Taken together, our results emphasize the importance of the mitochondrial apoptotic pathway and the non-genomic effects in GC-induced thymocyte apoptosis. The lower apoptotic sensitivity of $CD4^+$ T cells and Tregs can be the consequence that in these cells other non-genomic pathways get activated, but further investigations are needed to uncover the molecular background.

6. Summary of new results

1. We have observed that in DP thymocytes the activated GR co-localized with Bcl-2 proteins; Bax, Bak, Bcl-x_L and Bim, and we have also characterized how the rate of co-localisation changed after 30 min DX treatment.

2. We could have confirmed the co-localisation results with immunoprecipitation. Bak, Bcl-x_L and Bim precipitated with the GR, but Bax did not. We have also characterized how DX treatment changed the distribution of the proteins in the cytoplasm and mitochondria of the DP cells.

3. We have examined the subcellular distribution of Bax upon DX treatment. The Bax accumulated in the mitochondria.

4. We have observed that after 1 h DX treatment the level of cytochrome c in the cytoplasm significantly increased in DP thymocytes, as a consequence of the pore formation of pro-apoptotic proteins in the mitochondrial outer membrane.

5. We have described the activation of caspase-3, -8 and -9 after DX treatment. We could have detected significant increase after 2 h DX treatment. The activation of caspase-9 was more pronounced than caspase-8.

6. We could have confirmed the flow cytometry results with western blot. After 3 h of DX treatment the levels of activated caspases were significantly increased in the cytoplasm of DP cells.

7. We have observed significantly higher Annexin V level after 4 h of DX treatment in $CD4^+$ thymocytes. In CD4+ splenocytes, tTregs and pTregs the Annexin V positivity was much lower.

8. We have characterized the kinetics of caspases in thymic and splenic $CD4^+$ cells and in tTregs and pTregs. The tTregs have showed significant resistance to DX-induced apoptosis.

9. We have detected the co-localisation of GR and Foxp3 with confocal microscopy in tTregs and pTregs.

7. Publications

7.1 Publications related to the thesis (cumulative impact factor related to this thesis: 4,649)

1. Prenek Lilla, Ugor Emese, Papp Ramóna, Boldizsár Ferenc, Berki Tímea
   A glukokortikoid hormon nem genomikus hatásai a T-sejtek jelátvitelére és apoptózisára
   IMMUNOLÓGIAI SZEMLE 6:(3-4) pp. 54-58. (2014)

2. Prenek L, Boldizsar F, Kugyelka R, Ugor E, Berta G, Nemeth P, Berki T
   The regulation of the mitochondrial apoptotic pathway by glucocorticoid receptor in collaboration with Bcl-2 family proteins in developing T cells.
   APOPTOSIS 22:(2) pp. 239-253. (2017) **IF: 3,833**

3. Ugor E, Prenek L, Pap R, Berta G, Ernszt D, Najbauer J, Németh P, Boldizsár F, Berki T
   Glucocorticoid hormone treatment enhances the cytokine production of regulatory T cells by upregulation of Foxp3 expression
   IMMUNOBIOLOGY 223:(4-5) pp. 422-431. (2018) **IF: 2,702 (from this 0,816 IF is used to this thesis)**

7.2 Other publications:

Kugyelka R, Kohl Z, Olasz K, Prenek L, Berki T, Balogh P, Boldizsar F
Correction of T cell deficiency in ZAP-70 knockout mice by simple intraperitoneal adoptive transfer of thymocytes.
CLINICAL AND EXPERIMENTAL IMMUNOLOGY &: p. &. (2018) IF: 3,410

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