Thesis for the degree of doctor of philosophy (Ph.D.)

Studies on the enhancing effect of retinoids of glucocorticoid-induced apoptosis and on the mechanism of adenosine-induced apoptosis of mouse thymocytes

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I. Introduction

Apoptosis

Apoptosis – or programmed cell death – is a continuous physiological process in proliferating/regenerating tissues. Multicellular organisms need to get rid of those cells, which (a) were generated in an unnecessarily large amount; (b) do not play physiological roles; (c) have already done their duties; (d) mean potential threat to the life mechanisms; or (e) whose development does not take place properly. The cell removal is mediated via the process of apoptosis. Apoptosis is an active mechanism of cell elimination, in which damaged or unnecessary cells die, and their content is recycled via phagocytosis by macrophages. Cells undergoing apoptosis lose their connections to the extracellular matrix, their chromatin becomes condensed and gradually fragmented, irregular structure of cell-membrane and the shrinkage of the cytoplasm can be observed, however there are no essential alternations in other cell compartments. The contents of apoptotic cells are not released into the extracellular fluid, as a result no inflammatory response develops and cicatrisation is prevented. The mechanisms of programmed cell death act a central role in the development and maintenance of matured T-lymphocytes that are essential in cell-mediated immunity. Disorders during the apoptosis of T-cells can be responsible for numerous clinical patterns: enhanced apoptosis may cause leucopenia or immunodeficiency;

decreased programmed cell death can lead to the development of immune cell-originated tumors, while defective selection can contribute to the development of autoimmune diseases. Apoptosis-inducing agents are the so called 'apoptotic factors'. These can be ligands of cell surface receptors (e.g.: TNF or Fas ligand) or ligands of nuclear receptors (e.g.: glucocorticoid or retinoic acid), but decrease in the concentration or absence of survival factors (e.g.: erythropoietin, IL-1, CSF, IL-2, growth factors) and some other non-physiological effects (e.g.: UV radiation, toxins, hypoxia) can also lead to apoptosis. In addition, apoptosis can be the result of the combination of contradictory signals.

Development of T-lymphocytes and apoptosis

The generation of T-lymphocytes is initiated from hematopoietic stem cells of the bone marrow and takes place in the thymus, where the epithelial cells of the thymus, the dendritic cells and certain elements of the stroma (e.g.: fibroblasts) ensure essential microenvironment for the further maturation of T-cells. Those T-cells that show low affinity to MHC (major histocompatibility complex) molecules are supposed to die by neglection. Glucocorticoid hormone produced by the epithelia cells of the thymus accelerates the apoptosis of the neglected cells. In the course of negative selection, those T-lymphocytes that recognize their self-MHC protein peptide complexes by high affinity also undergo apoptosis. Only those thymocytes will survive and leave the thymus,

which possess limited ability to recognize self-antigens, therefore do not mean potential threat to their own tissue.

The glucocorticoid hormone

Glucocorticoid hormone is a member of steroid hormone family, regulates several essential biological processes, and also plays an important role in the differentiation, activation, survival and apoptosis of T-cells. This hormone is generated endogenously and acts in the regulation of inflammation. Glucocorticoids are one of the most frequently used drugs due to their strong anti-inflammatory, immunosuppressive and lymphocyte apoptosis-inducing effects. In spite of its advantageous medical effects, their therapeutical utilization is restricted or has a time-limitation because of the side effects such as osteoporosis or skin atrophy. Not only the adrenal cortex, but also the cortico-epithelial cells of the thymus are able to produce the glucocorticoid hormone, therefore glucocorticoids have also a local effect on the thymocytes.

The Glucocorticoid Receptor (GR)

The glucocorticoid hormone mediates its effects by binding to the glucocorticoid receptor, which acts in the nucleus of the cell. Steroid receptors belong to the superfamily of ligand-induced transcription factors. These receptors are composed of several identical structural units: one conserved, central Zn-finger DNA-binding domain

(DBD), one less conserved C-terminal ligand-binding domain (LBD) and one less conserved N-terminal domain (NTD). Although the vast majority of steroid receptors can be found in the nucleus, in the absence of the hormone, GR stays in the cytosol in complexes with various regulatory proteins (such as Hsp90, Hsp70, Hsp56). The complex of GR-Hsp90 not only ensures the proper conformation of the receptor, but also prevents the receptor from binding to the DNA in the absence of the ligand. Following glucocorticoid hormone binding, the GR and the heat shock protein dissociate, the GR undergoes dimerization, and translocates into the nucleus. In the nucleus, the homodimer receptor recognizes strongly conserved palindrome sequences (GGRACAnnnTGTTCT) in the promoter of various genes and binds to these specific DNA sections called 'glucocorticoid response elements' (GRE). Following DNA binding, the ligated GR enhances or inhibits the transcription of numerous genes. Transcription of several genes is known to be enhanced by glucocorticoids including those, which take part in the activation or apoptosis of T lymphocytes (such as GILZ).

Retinoic acids and retinoid receptors

Vitamin A is an essential microelement for the proper differentiation of epithelial tissue, in the mechanism of vision and for reproduction; furthermore it plays a basic role in the maintenance of adaptive and innate immunity. Vitamin A deficiency has been known for a long time to be accompanied with immune deficiency and susceptibility to a

wide range of infectious diseases. In vitamin A deficient animals, a marked atrophy of the thymus and the spleen has been observed. On the other hand, high doses of retinoids are toxic and cause involution of lymphoid organs, particularly the thymus, whereas moderate subtoxic doses of retinoids have been reported to result in a significant increase in thymus weight, numbers of thymic small lymphocytes, and in cellularity of the lymph nodes. The effect of Vitamin A on the immune system is primary mediated by its active metabolites, the retinoic acids: *trans*- and 9-*cis*-retinoic acids. Retinoic acids are the ligands of the nuclear retinoid receptor family. Trans-retinoic acid (ATRA) and 9-cis-retinoic acid (9cRA) can be found in numerous types of tissue, their effects are based on the ligation of their specific receptors, the retinoid receptors. Retinoid receptors, similar to GR, belong to the nuclear steroid/thyroid/retinoid hormone receptor superfamily, and regulates the expression of vatrious target genes by binding to specific responsive elements (RARE, RXRE). In addition, they can also interact with other transcription factors and regulate their functions. Two types of retinoid receptor have been described: retinoic acid receptors (RAR) and retinoid X receptors (RXR). ATRA and 9cRA are equipotent in activating RAR, while activation of RXR is mediated only by 9cRA. Both RAR and RXR have three subtypes: α, β and γ. In the presence of RAs, retinoid receptors function in the form of RAR/RXR heterodimers or RXR/RXR homodimers. In addition, RXR can form heterodimers with various other members of the steroid/thyroid/retinoid receptor family, including thyroid hormone receptor, Vitamin D receptor, peroxisome

proliferator-activated receptor, pregnan X receptor, constitutive androstane receptor and some orphan transcription factors such as Nur77. The effect of retinoic acid in the aspect of the regulation of the immune-system is highly complex. In physiological concentrations they inhibit the proliferation of B-cells and their precursors. In contrast, the proliferation of T-cells is enhanced by RAs via promoting the TCR-induced IL-2 production. Furthermore ATRA induces the transformation of FoxP3- CD4+ T-cell into FoxP3+ regulatory T-cells. Several years of experimental work in our research lab have revealed that RAs also play a role in the regulation of T-lymphocyte development within the thymus.

Adenosine and adenosine receptors

Adenosine is a generally occurring nucleoside in living organisms. Under physiological conditions it is continuously degraded within the cells, however its production is elevated following tissue injuries or under hypoxia. Increases in the endogenous levels of adenosine are linked to cytoprotective, anti-inflammatory and pro-inflammatory mechanisms, but apoptotic functions of adenosine have also been described. In the thymic environment adenosine is present in a relatively high concentration, because it is continually secreted by macrophages that engulf apoptotic thymocytes. During the regulation of normal immune mechanisms, adenosine binds to its receptors on the cell surface, and this way it is capable of inducing apoptosis in both human and mouse thymocytes and in activated T-

cells. The stimulatory or the inhibitory effect of adenosine on various cellular functions depends on the type of the activated adenosine receptor. In vertebrates the adenosine receptors belong to the purinergic receptor family. The endogenous ligands of these receptors are adenosine, AMP, ADP and ATP. According to their sensitivity to adenine-nucleoside (adenosine) or nucleotide (ATP) purinergic receptors are divided into P1 and P2 groups. P1 receptors are identical to adenosine receptors. The latter can be divided into four subgroups: A_1, A_{2A}, A_{2B} and A_3. All of them are G protein coupled receptors. The actual cell response for the receptor activation is developed as a result of the activation of the adenylate cyclase signaling pathway and the phosphorylation of further proteins. Moreover, adenosine receptors can also activate the phospholipase C (PLC) signaling pathway. Enhanced activity of PLC leads to Ca^{2+} release from the intracellular stores via opening the inositol triphosphate (IP_3) calcium channels. The increased adenosine concentration and the resulted T- and B-cell immune deficiency observed in patients lacking adenosine deaminase focused the attention to the strong thymocyte-killing effect of adenosine. Experimental studies have proven that this killing effect of adenosine is mediated by A_{2A} receptors, but the mechanism of killing has not been clarified.

II. Purposes

Previous research in our laboratory has shown that retinoids are produced in the thymic environment, and are capable of both inducing apoptosis of the neglected cells, and of enhancing apoptosis thymocytes induced by the glucocorticoid hormone. Studies in our laboratory have also demonstrated that macrophages engulfing apoptotic cells secrete adenosine, and adenosine can induce apoptosis in thymocytes. Other research groups have shown that this effect of adenosine is mediated via the adenosine A_{2A} receptor.

Based on these results, in my experiments I intended to examine two fields:

1. By what kind of mechanisms do retinoids enhance the glucocorticoid-induced apoptosis of thymocytes?
2. What is the mechanism of the adenosine A_{2A} receptor-mediated cell death in mouse thymocytes?

III. Experimental animals

The following mice were used in the experiments: 4 week-old NMRI mice, A2A-/-, Nur77-/-, Bim-/- knock out, FADD-dominant, *vav-bcl-2-269*, *Eμ-bcl-2* transgene mice and their wild type counterparts.. Except from NMRI mice, all the mice were generated on a C57/BL6 background.

IV. Methods

Characterization of thymocyte populations and detection of apoptosis

For *in vivo* induction of apoptosis of thymocytes, 4 week-old mice were injected intraperitoneally by 0.2 mg dexamethasone-acetate alone or by simultaneously with 50 μg AM580 or with 50 μg LG268 dissolved in 0.1 ml ethanol and 0.4 ml physiological saline. For testing the effect of adenosine *in vivo*, we injected various concentrations of NECA. After 24 hours of different *in vivo* treatments, thymocytes were isolated from the thymus, suspended in ice-cold PBS containing 0.1 % Sodium-azide (Na-azide) before staining with PE-labeled anti-CD4 and FITC-labeled anti-CD8 antibodies. Cells were incubated on 4°C for 30 minutes, then washed twice, once with 1% BSA, and once with ice-cold PBS containing 0.1 % Na-azide. The doubled fluorescence was detected on 488 nm

wavelength using Becton Dickinson FACScan. Unstained thymocytes served as anti-fluorescence control.

During the apoptosis analysis thymocytes or the T-cell lines were treated by dexamethasone (1μM) and different retinoids for 6 hours in the presence of 10% delipidated FCS, in case of the adenosine or adenosine analogs in 10% FCS. In the case of the thymocytes, the rate of the DNA degradation by the effect of the treatments was determined by diphenylamine reagent. In the case of the T-cell lines the determination of the percentage of the cells that contain degraded DNA was carried out by flow-cytometric analysis after staining the cells by propidium-iodide (PI). For further verification of the apoptosis induction we have used combined Annexin V and pI staining.

Detection of the expression of Nur77, FasL, NDG-1 and -2, Bim genes by RT-PCR

4 hours after treatments, mRNA expression levels of the genes listed above were determined by RT (reverse transcription)-PCR. After isolating the total RNA by Trizol, mRNAs that carry polyA tails were reverse transcribed to single-stranded cDNA by the help of oligodT primers and reverse transcriptase enzyme, and then PCR reaction was carried out according to given parameters. PCR products were analyzed by agarose gel-electrophoresis.

Quantitative analysis of mRNA expression of GILZ, Nur77, FasL, NDG-1 and -2, Bim and TRAIL genes by Q-PCR

Total RNA from the cells was isolated by RNeasy® Mini Kit. The next step was reverse transcription (RT), where we have used 100 nM total RNA for the synthesis of cDNA per sample. The RT reaction was carried out on 42°C for 30 minutes, then on 72°C for 5 minutes using specific reverse primer and Superscript II Reverse Transcriptase Kit (Intvitrogen, Carlsbad, USA). After RT the amount of transcripts was determined by using Taqman probes. The assay was performed using ABI Prism 7900 device, according to the following program (40 cycles): 94°C for 12 seconds, then 60°C for 1 minute. The results were normalized for the housekeeping gene cyclophyllin A.

Transient transfection

COS1 cells were plated on a 48 well plate 24 hours prior to transfection (10^5 cells/well). Next day the transient transfection of the cells was carried out by using PEI (polyethylene-imine). The cells were transfected by pCMX-GRE-luc plasmid alone or simultaneously by pCMX-FL-hRARα and/or pCMX-FL-hRXRα plasmids. The reporter plasmid was also transfected into the cells in the same amount as the previous constructs. After 5 hours of incubation, cells were treated by given concentrations of dexamethasone and retinoids for 48 hours. After the treatment, cells

were lysed. The activity of luciferase enzyme was determined using Luciferase Assay system Kit. The enzyme activity results were normalized for the β-galactosidase activity used as control.

Co-immunoprecipitation

Thymocytes ($4x10^7$) were treated by dexamethasone (1μM) alone or simultaneously with different retinoids for 1 hour in RPMI 1640 medium containing 10% delipidated FCS. Following the treatments, cells were lysed and GR was immunoprecipitated by adding agarose-conjugated anti-mouse GR antibody. After SDS gel-electrophoresis the immunoprecipitated GR and the co-immunoprecipitated RARα receptor were identified by Western blot analysis using anti-mouse GR and anti-mouse RARα antibodies. Possible alterations in the phosphorylation of Ser232 of GR after retinoid treatments were also tested on immunoprecipitated GR. In this case, a specific antibody was used against the phosphorylated Ser232.

Mammalian Two-Hybrid System

293T fibroblast cells were transfected by pCMX-Gal-L-hGR and pMH100-TK-luc plasmids alone or simultaneously with plasmids expressing VP-hRARα-LBD or VP-hRXRα-LBD. pCMX-β-galactosidase plasmid was used as control for normalization. The process of the experiment and the measurements of the luciferase

activities were carried out similarly to the experiments of transient transfections.

Determination of the intracellular concentration of cAMP

Following the different treatments the intracellular concentration of the cAMP was determined by the use of cyclic AMP Enzyme Immunoassay Kit, according to the User's manual of the kit. We analyzed 10^6 cells per sample.

Determination of the cytosolic Ca^{2+} concentration in the thymocytes

Thymocytes were treated in RPMI 1640 media (0.2 mM L-glutamine, 5% FCS) containing 5 µl Fura-2-AM (Fura-2-acetoxymethyl-ester) for 1 hour in room temperature. After treatment, cells were washed to remove Fura-2-AM and were suspended in fresh media. The rate of the alternation in the cytosolic Ca^{2+} concentration was determined by spectrophotometry on 340/380 nm wavelength using Quartz cuvette.

V. Results

Ligation of RARα and RXR promotes dexamethasone-induced apoptosis in mouse thymocytes *in vivo* and *in vitro*

In the course of our experiments we have examined the dose-dependent effect of dexamethasone on the apoptosis of mouse thymocytes. Dexamethasone induced apoptosis in a dose-dependent manner in thymocytes after 6 hour-long treatments. For further experiments, 0.1 μM concentration of dexamethasone (inducing apoptosis in 50% of the cells) was selected to test the potential enhancing or inhibiting effect of retinoids. The following retinoids were applied: ATRA a pan RAR agonist; 9cRA a pan RAR and RXR agonist; AM580 and CD2081, RARα agonists, CD2503, an RARα antagonist; CD437, CD666 and CD2325 RARγ agonists, and LG268 an RXR agonist. It was found that all ligands, which were specific either for RARα or for RXR, efficiently increased the rate of dexamethasone-induced apoptosis in thymocytes by about 30%. Ligands, which act on RARγ, were proven to be ineffective. ATRA enhanced glucocorticoid-induced apoptosis in thymocytes less efficiently than 9cRA, however when LG268 (0.1 nM) was added together with ATRA, the two compounds promoted glucocorticoid-induced apoptosis more efficiently than 9cRA alone. In our further studies we tested the impact of retinoids on thymocytes isolated from RARα knock-out mice. In the absence of RARα only compounds activating RXR were able to enhance glucocorticoid-induced cell

death. For further verification of the role of RARα, we tested the effect of an antagonist (CD2503) of RARα. In case of wild-type dexamethasone-treated cells, CD2503, also increased the apoptosis rate, but had no effect on RARα knock-out thymocytes. These observations suggest that CD2503 acted indeed via RARα, and the transcriptional effect of retinoid receptors is not required for enhancing the glucocorticoid-induced death. Besides the *in vitro* experiments, we have carried out 24 hours long *in vivo* treatments as well, by injecting 0.2 mg dexamethasone alone or together with 50 μg retinoid intraperitoneally. Ligation of both RARα and RXR enhanced the GR-induced apoptosis of the thymocytes als *in vivo*, and mainly the CD4+CD8+ double positive population was affected.

Retinoids enhance the GR-induced expression of GILZ (glucocorticoid-induced leucine zipper), during thymocyte apoptosis

Based on the previously described fact that GR-induced apoptosis depends on the transcriptional activity of GR, we decided to test whether retinoids affect the transcription of GR-induced genes. During dexamethasone-induced cell death enhanced expression of several genes has already been described. We have selected to test the expression of GILZ, which is activated during the early stage of GR-induced apoptosis, and its promoter carries numerous GREs. Isolated thymocytes were treated by dexamethasone alone or together with various retinoids. Following the 2 hours treatments

GILZ mRNA expressions were detected by qPCR technique. It was found that both the applied RARα agonist and antagonist, and also the agonist of RXR enhanced the GR-induced gene-expression level of GILZ in a dose-dependent manner.

RARα/RXR heterodimers mediate the trans-activating effects of retinoids

To analyze further the trans-activating effect, an *in vitro* glucocorticoid luciferase reporter assay was applied, in which cells that express retinoid receptors at low level, were transiently transfected by pCMX-GRE-luc reporter plasmid, and by pCMX-FL-hRARα and/or pCMX-FL-hRXRα vector constructions. In physiological concentration, ATRA had no effect on GR-induced transcription in the presence of RXR and have shown quite low transcriptional induction is the presence of RARα or RARα/RXR. 9cRA significantly enhanced GR-induced transcription both in the presence of RXR and RARα. However, the effect of 9cRA was much more pronounced, when RARα/RXR were collectively expressed in the cells. The RXR agonist L268 also significantly increased the transcription in the presence of both RXR and RARα/RXR. AM580 and CD2503 were ineffective, when only RXR was present, and only slightly elevated the GR-induced transcription, when only RARα was expressed. However, when both retinoid receptors (RXR and RARα) were expressed, both RARα ligands enhanced the GR-induced transcription. When cells expressing RARα/RXR were treated with AM580 and LG268 simultaneously, a much more

efficient transcription could be detected. These findings indicate that RARα/RXR heterodimers mediate the effect of retinoids on GR-induced transcription, and the effect is more pronounced, when both sides of the receptor are ligated. The fact, that CD2503, which does not enhance the transcriptional activity of RARα, possessed similar activity as the receptor agonist, indicates that it is not the trans-activating effect of the retinoid receptors by which they enhance the function of GR. Therefore we tested using DNA-binding mutants of the retinoid receptors, whether DNA binding is required for the action of retinoid receptors. Our data indicate that binding of the receptors to the DNA is not necessary for achieving the observed effect.

Glucocorticoid and retinoid receptors interact following binding of ligand

It is known that following ligand binding nuclear receptor can activate not only their own genes, but they can influence the transcriptional activity of other transcription factors as well, by interacting with them directly. Binding of the nuclear receptors to the DNA is not necessary for this interaction. Therefore we tested a possible interaction between the retinoid receptors and GR. When thymocytes were treated simultaneously with dexamethasone and retionoids, RARα co-immunoprecipitated with the GR. The fact that

LG268, which is an RXR ligand, also induced interaction between RARα and the ligated GR indicated that within the thymocytes LG268 must bind to RARα/RXR heterodimers. We used mammalian two-hybrid system to test whether the interaction between the receptors is established directly. 293T fibroblast cells were transfected by pCMX-Gal-L-hGR and pMH100-TK-luc plasmids alone or simultaneously with the VP-hRARα-LBD plasmid. In those cases, when VP-hRARα-LBD was also present in the cells, a similar luciferase activity could be detected, as in the case of dexamethasone treatment, but in the absence of VP-hRARα-LBD plasmid. This result indicates that the receptors might interact directly. Since ligation of RXRα in the presence of RXRα alone could promote dexamethasone-induced transcription in the transient transfection assay, using again the two mammalian hybrid technique we checked, whether RXRα can also interact with GR. In these experiments a similar luciferase activity could be detected in the cells transfected simultaneously by pCMX-Gal-L-hGR /pMH100-TK-luc plasmids and VP-hRARα-LBD plasmid, than that was measured in the case of dexamethasone treatment in the absence of VP-hRARα-LBD

plasmid. In the presence of the ligands of both receptors the interaction became more efficient detected by the enhanced luciferase activity. These data imply that strong interaction between GR and RXRα is induced by simultaneous ligand binding of both receptors.

Retinoids do not affect the Ser232 phosphorylation of GR

It was previously reported that the phosphorylation of Ser232 increases the transcriptional activity of GR in a promoter-specific manner. That is why we decided to test whether retinoid-treatment affects dexamethasone-induced phosphorylation of Ser232 in mouse thymocytes. During the experiment, thymocytes were treated for one hour by 0.1 μM dexamethasone alone or simultaneously with 0.3 μM AM580, CD2503 or 50nM LG268. Although the addition of dexamethasone enhanced phosphorylation of Ser232 on GR, retinoids have no effect on it indicating that ligated retinoid receptors must act by a different mechanism.

Adenosine induces adenosine A_{2A} receptor-dependent and -independent cell death in mouse thymocytes *in vitro*

Previous experiments based on A_{2A} receptor antagonists have suggested that the A_{2A} receptor of adenosine mediates the adenosine-induced cell death in mouse thymocytes. Based on these findings in the first part of our studies we tested the effects of adenosine and different adenosine analogues on the thymocytes from wild-type and

adenosine A_{2A} receptor knock-out mice. The results of these experiments showed that while adenosine enhanced the apoptosis of wild-type thymocytes in a dose-dependent manner, adenosine below 200 μM concentration did not induce apoptosis in the A_{2A} receptor null thymocytes. The same phenomenon was observed when cells were treated by adenosine analogues. Both NECA (a non-specific A_{2A} receptor agonist) and DPMA and CGS21680 (adenosine A2A receptor-specific agonist) induced dose-dependent apoptosis in wild-type thymocytes. These compounds at low concentrations did not cause apoptosis in A_{2A} knock-out thymocytes. Thymocytes were treated also by an adenosine A_{2A} receptor antagonist (ZM41285) administrated simultaneously with adenosine or NECA. The antagonist in the presence of 100 μM adenosine or 25 μM NECA fully inhibited the apoptosis-inducing effect. To exclude the possibility that the lack of apoptotic response of A_{2A} knock-out thymocytes to these treatments is related to a defect in the apoptosis machinery, thymocytes lacking A_{2A} receptors were treated with compounds that induce apoptosis via other signaling pathways. However to these stimuli A_{2A} receptor knock-out thymocytes responded as well as wild type cells indicating that the lack of response was related to the adenosine receptor. These findings together confirm that adenosine induces A_{2A}-mediated apoptosis in mouse thymocytes.

Adenosine A_{2A} receptor activates the adenylate cyclase signaling pathway in mouse thymocytes

Since adenosine A_{2A} receptors are known to activate both adenylate cyclase and phospholipase C signaling pathways, we intended to clarify which signaling pathway mediates the effect of adenosine in mouse thymocytes. Thymocytes were treated for 15 minutes by adenosine and various adenosine analogues, and the amount of the generated cAMP was determined by 'cyclic AMP Enzyme Immunoassay Kit', while alternation in the intracellular Ca^{2+} levels were detected by Fura-2AM (membrane permeable derivate of the calcium indicator Fura-2). We have found detectable increases in the intracellular cAMP levels, but no elevation in cytosolic Ca^{2+} concentration could be detected. However, compounds acting on the same adenosine receptors were not capable of inducing cAMP elevation in A_{2A} knock-out thymocytes indicating that adenosine acting on A_{2A} receptors activates the adenylate cyclase signaling pathway.

Adenosine stimulates the expression and DNA binding of Nur77 *in vitro* in wild-type thymocytes by regulating the level of intracellular cAMP

To test whether adenosine-induced apoptosis needs the appearance of new proteins, we have applied actinomycin D and cycloheximide treatment. The former compound inhibits the synthesis of RNA

while the latter one is an inhibitor of protein-synthesis. Both actinomycin D and cycloheximide treatments inhibited completely the adenosine- or dbcAMP-induced apoptosis suggesting that adenosine-induced apoptosis of thymocytes is dependent on intact transcriptional and translational mechanisms. Since it has been previously described that Nur77 plays an important role in the regulation of the negative selection of thymocytes, and its expression can be enhanced by the adenylate cyclase signaling pathway in neurons, we tested the potential involvement of Nur77 in the regulation on adenosine-mediated cell death. We have observed the induction of Nur77 following 4 hours *in vitro* treatment by adenosine in case of wild-type thymocytes, while this induction could not be detected in the receptor knock-out cells. Furthermore, elevation in the levels of cAMP induced by forskolin (activator of adenylate cyclase), cholera toxin (G_s activator) and dbcAMP also lead to increased Nur77 expression. Nur77 could be detected in numerous protein bands, suggesting that it is not only induced, but is also phosphorylated following activation of the adenylate cyclase pathway.

Nur77 and several Nur77-dependent genes are induced in wild-type, but not in $A_{2A}^{-/-}$ thymocytes following *in vivo* injection of NECA

Next we tested Nur77 induction by adenosine also under *in vivo* conditions. After injection of NECA, Nur77 was induced in a dose-

dependent manner in wild-type mice, in contrast to knock-out mice, where the induction could not be observed. We found that Nur77-dependent (FasL, TRAIL and NDG-1,-2 apoptosis) genes are also detectable following treatments by NECA. The expression of these genes was again restricted to the wild-type cells. Since our preliminary studies in the aspect of negative selection have suggested that the induction of Bim (a BH3-only protein) might be related to the transcriptional activity of Nur77, we have examined the level of Bim expression following injection of NECA. Our results showed that among the two forms of Bim, Bim_{EL} (extra-long) and Bim_{L} (long), the latter was induced following NECA injection. But it could be observed again only in wild-type thymocytes.

Bim, a BH3-only protein, mediates the apoptosis-inducing effect effect of adenosine in mouse thymocytes

Next it was tested, which of the molecules regulated by adenosine has a determinant role in the induction of apoptosis. The role of Nur77 was examined in Nur77 knock-out thymocytes. The action of FasL and TRAIL were neutralized by FasFc chimera and soluble DR5 molecule, respectively. The vast majority of apoptosis receptors activate the procaspase 8 enzyme through the FADD molecule. The effect of FADD and the effect of apoptosis receptors are inhibited in mice expressing the dominant negative protein. Our results showed that neutralizing the effect of these molecules did not inhibit, but enhanced adenosine-induced apoptosis. Thus despite of the fact that

adenosine promotes the expression of these molecules, none of these proteins mediate in the observed cell death. Then the role of Bim was addressed. During the experiment we used three sets of thymocytes: one that lacks the Bim protein, and two other types that over-expresses Bcl-2, an anti-apopototic protein, that counteracts the action of Bim. We have noticed that both the lack of Bim and the over-expression of Bcl-2 inhibit adenosine-induced apoptosis suggesting that the adenosine A_{2A} receptor induces thymocyte apoptosis via upregulating the proapoptotic Bim.

VI. Discussion

In this present study we examined the mechanism by which retinoids enhance the GR-induced cell death, and the mechanism through which adenosine causes apoptosis in mouse thymocytes.

Still ongoing investigations in our laboratory have shown that there are several molecules in the thymic microenvironment that contribute to the apoptosis induction of neglected cells. These molecules include retinoids which are produced by both thymic epithelial cells and engulfing macrophages, and adenosine which is secreted by engulfing macrophages.

In this present study we have strengthened our previous observation, which showed, hat retinoids are capable of enhancing GR-induced apoptosis in immature thymocytes. Applying various retinoids and

RARα deficient thymocytes, we have proven that the apoptosis-enhancing effect of retinoids is mediated via RARα /RXR heterodimers. Since an applied RARα-specific antagonist also enhanced the GR-induced apoptosis, we suggested that it is not the transcriptional activity of the retinoid receptor that is required for enhancing GR-induced apoptosis.

We have shown that in thymocytes retinoids enhance the expression of the glucocorticoid-inducible gene, GILZ through RARα / RXR, thus they can enhance the transcriptional activity of GR. This mechanism was further verified in experiments, where the induction of the glucocorticoid-dependent induction of the luciferase reporter enzyme was studied in the presence of various retionoid receptors. In the presence of the RARα/RXR heterodimer, all the examined agonists of RAR and RXR, and the RARα antagonist itself were able to increasethe transcriptional activity of GR. Presence and ligation of RARα alone was not sufficient in enhancing the transcriptional activity of GR, while ligation of RXR in the presence of RXR alone, or in RARα/RXR heterodimer form was sufficient to enhance it. These data together indicate a determining role of RXR in the regulation of GR-induced transcription acting alone or after forming a heterodimer with RARα.

Studying the possibility of direct interaction between GR and retinoid receptors by mammalian two-hybrid system, we have proven that GR and RARα or RXR are connected to each other. However while this interaction was not promoted by the ligation of

RARα, ligation of RXR ligand strongly enhanced it. The interaction between RARα and GR was also confirmed by co-immunoprecipitating the RARα and the GR. However these receptors could be detected together only, when thymocytes were simultaneously treated by dexamethasone and those retinoids, including the RXR agonist LG268 that increased the glucocorticoid-induced apoptosis. According to this latter observation it seems that although LG268 could act through RXR homodimers, yet it functions through RARα/RXR heterodimers *in vivo*. Our data altogether imply that the transcriptional promoting effect of GR is mediated by the RXR of the RARα/RXR heterodimer, and the conformation that is needed for the interaction with GR can be stabilized by the ligation of both sides of the RARα/RXR heterodimer.

During the examination of the role of adenosine in thymocyte apoptosis we have proven that adenosine induces cell death via adenosine A_{2A} receptors. Since it was known that adenosine A_{2A} receptors can activate both the adenylate cyclase and phospholipase C signaling pathways, we tested that in mouse thymocytes which pathway is involved in the initiation of the apoptosis. Our results demonstrated the involvement of adenylate cyclase and cAMP-dependent protein kinases. Our data also confirmed that the adenosine-mediated cell death depends on the intact transcriptional and translational activity of the cells, during which Nur77 and Nur77-dependent genes are also induced. The observed effects were

missing in the case of A_{2A} knock-out thymocytes, proving the role of A_{2A} receptor in the induction of Nur77. Interestingly, though Nur77 was previously shown to mediate negative selection, it was not required for adenosine-induced cell death.

Since it was also known in connection with the negative selection of thymocytes that besides Nur77, Bim is also induced, we tested if there is a change in the expression of Bim in the course of adenosine-induced cell death. Our results show that Bim is induced by adenosine, and again only in wild-type thymocytes. While Nur77 was not needed for adenosine induced cell death, Bim null cells were resistant. In addition, overexpression of Bcl-2 – an antagonist of Bim –also prevented adenosine-induced cell death. These data together suggest that thymocyte apoptosis mediated by adenosine A_{2A} receptors is regulated through a pathway, which involves Bim.

Our results have two medical consequences. On one side we found that retinoids enhance glucocorticoid-induced cell death not only in thymocytes, but also in glucocorticoid-sensitive malignant T cells, which poses its utilization in the treatment of glucocorticoid sensitive leukaemias. On the other hand, the thymocyte-killing effect of adenosine could offer possible explanation for the immunodeficiency of patients missing the adenosine deaminase enzyme, where the defect in the degradation of adenosine results high adenosine concentration in the environment of the cells.

VII. Summary

Time-dependent induction of apoptosis has an important role in normal cell cycle and development. During the selection and the differentiation of T-lymphocytes in the thymus, 90% of the thymocytes die due to negative selection and neglection. The apoptosis-inducing role of glucocorticoid hormone in the latter mechanism was proven. Thymic epithelial cells are capable of producing not only glucocorticoid hormone, but also retinoids, and the involvement of retinoids was also verified in the process of both apoptosis induction and in the enhancement of glucocorticoid-induced cell death.

Besides glucocorticoids and retinoids, it was observed that adenosine produced by macrophages engulfing apoptotic thymocytes also plays a regulatory role in the process of thymocyte apoptosis acting through the adenosine A_{2A} receptors.

In my studies it was shown that retinoids enhance glucocorticoid-induced apoptosis of mouse thymocytes by ligating the RARα/RXR heterodimer. The ligated RARα/RXR heterodimer directly interacts with the ligated GR. The interaction between the two nuclear receptors does not require the DNA binding of the RARα/RXR heterodimer, and promotes the transcriptional activity of GR. The mechanism of this enhancing effect was not clarified yet, but it

involves the RXR side of the RARα/RXR and does not involve alternations of the phosphorylation state of GR.

We have also described that thymocytes can die following stimulation of the adenosine A_{2A} receptor, which leads to the activation of the adenylate cyclase system and induction and phosphorylation of Nur77, appearance of Nur77-dependent genes and the appearance of Bim. While adenosine-induced death did not require Nur77, Bim, the BH3 only proapoptotic protein, was found to play an essential role in inducing it.

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List of publications related to the dissertation

1. **Tóth, K.Á.**, Sarang, Z., Scholtz, B., Brázda, P., Ghyselinck, N., Chambon, P., Fésüs, L., Szondy, Z.: Retinoids enhance glucocorticoid-induced apoptosis of T cells by facilitating glucocorticoid receptor-mediated transcription.
Cell Death Differ. Epub ahead of print (2010), 10.p, 10.p.
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2. Kiss, I., Kovácsné Oskolás, H., Tóth, R., Bouillet, P., **Tóth, K.Á.**, Fülöp, A., Scholtz, B., Ledent, C., Fésüs, L., Szondy, Z.: Adenosine A2A receptor-mediated cell death of mouse thymocytes involves adenylate cyclase and Bim and is negatively regulated by Nur77.
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