Retinoids induce Nur77-dependent apoptosis in mouse thymocytes

Beáta Kiss a,1, Katalin Tóth a,1, Zsolt Sarang b, Éva Garabuczi a, Zsuzsa Szondy a,⁎

a Department of Dental Biochemistry, Signaling and Apoptosis Research Group, Research Center of Molecular Medicine, University of Debrecen, Debrecen H-4012, Hungary
b Department of Biochemistry and Molecular Biology, Signaling and Apoptosis Research Group, Research Center of Molecular Medicine, University of Debrecen, Debrecen H-4012, Hungary

1. Introduction

Vitamin A deficiency has been known for a long time to be accompanied by immune deficiency and susceptibility to a wide range of infectious diseases [1] due to the various effects of vitamin A on the immune functions [2]. Suggestions have been made that the active metabolites of vitamin A, that mediate its effects on the immune system, are the retinoid acids (RAs)—all-trans RA (ATRA) and 9-cis RA (9cRA) [3,4]. Among many others, retinoids were shown to be produced in the thymus [5–9], and to induce apoptosis in immature thymocytes [9–12].

ATRA and 9cRA are suggested natural ligands for the nuclear retinoic acid receptor family [13]. ATRA and 9cRA are equipotent in activating retinoic acid receptors (RARs), while activation of retinoid X receptor (RXR) by ATRA is 50-fold less than by 9cRA [14]. Though ATRA does not bind to RXR receptors, the latest observation is explained by conversion of the ATRA to 9cRA within the cells reaching an intracellular concentration sufficient to activate RXR receptors at high extracellular concentrations of ATRA [15]. In the presence of RAs retinoid receptors function in the form of RAR/RXR heterodimers or RXR/RXR homodimers [16], and modulate gene expression either by direct binding to their cognate response elements or via interaction with other transcription factors. In addition, RXR can form heterodimers with various other members of the steroid/thyroid/retinoid receptor family, including Nur77 [17].

Nur77 also belongs to the steroid/thyroid hormone receptor superfamily, and is an orphan receptor for which no ligand is known [18]. Besides forming a heterodimer with the retinoid X receptor to confer 9cRA-dependent transcription to reporters containing the DR5 regulatory element, it can also bind in monomeric form to promoters containing the Nur77 binding response element [19], as well as a homodimer to the Nur77 response element carrying ones [20]. Nur77 was shown to be a determinant transcription factor in mediating T cell receptor-induced cell death of both thymocytes (negative selection) and peripheral T cells or T cell lines [11,21–24]. It was shown to be induced during T cell receptor-induced death of T cells, and the binding of it to its response elements correlated well with the onset of apoptosis. In addition, constitutively active Nur77 was found to induce thymocyte apoptosis [25,26]. To identify globally the targets of Nur77 transcription that regulate apoptosis in thymocytes, differential gene expression experiments were conducted with RNA isolated from Nur77 transgenic and wild-type fetal thymi with cDNA microarrays. These studies identified Fas ligand (FasL) and TRAIL, two known death receptor ligands, and Nur77 dependent...
gene-1 coded protein (NDG-1) that regulates Caspase 8 activity by unknown mechanisms, as mediators of Nur77-dependent apoptosis [27].

Studies done on cancer cell lines revealed first, that in addition to regulating transcription, Nur77 is also able to induce apoptosis independent of its transcriptional activity by targeting mitochondria, where it can convert Bcl-2, an antiapoptotic protein into a proapoptotic molecule. This effect is achieved via its association with the linker region of Bcl-2 between the BH3 and BH4 domains resulting in the exposure of the Bcl-2 BH3 domain [28]. In thymocytes during negative selection Nur77 also translocates into the mitochondria and contributes to the apoptosis induction [29]. This mitochondrial targeting is regulated by protein kinase C [30] and requires heterodimerization with RXRα, which carries a putative nuclear export sequence present in its carboxyl-terminal region [31].

Previous studies in our laboratory have shown that retinoids induce a transcription-dependent apoptosis in thymocytes via activating RARy [12]. However, the mechanism of the apoptosis induction was not investigated so far. Our data presented in this paper demonstrate that retinoids induce a Nur77-dependent apoptosis program in thymocytes involving both transcription-dependent and -independent mechanisms mediated by Nur77.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma-Aldrich (Budapest) except indicated otherwise.

2.2. Thymocyte suspensions

Thymocyte suspensions were prepared from thymus glands of 4 week-old Nur77+/+ mice and their Nur77 deficient [32] littersmates by mincing the glands in RPMI 1640 media supplemented with 10% charcoal-treated FCS, 2 mM glutamine, and 100 IU penicillin/100 μg streptomycin/ml. In a few experiments thymocytes were isolated from STAT1−/− mice (Jackson Laboratories [33], as well. Thymocytes were washed three times and diluted to a final concentration of 5 × 10⁶ cells/ml before incubation at 37 °C in a humidified incubator under an atmosphere of 5% CO2/95% air. Cell death was measured by trypan blue uptake. A total of 95–98% of cells routinely excluded trypan blue after the isolation procedures. These studies have been reviewed and approved by the review committee of the University of Debrecen (DEMÁB).

2.3. Determination of the percentage of apoptotic thymocytes

Thymocytes were incubated in 24 well plates in the presence of the indicated concentrations of retinoids for 12, dexamethasone-acetate (0.1 μM) or J02 antibody (1 μg/ml) for 6 h. To test the involvement of caspases thymocytes were preincubated with 40 μM z-IETD.fmk (BD Pharmingen) or 75 μM z-LEHD.fmk Calbiochem (San Diego, CA, USA) for 1 h. For determining the amount of cells containing degraded DNA characteristic for apoptosis, a rapid technique using propidium iodide DNA staining was applied. For staining cells were fixed with 70% ice cold ethanol for 5 min, then washed and resuspended in 100 μl PBS containing 100 μg/ml RNase and incubated for 10 min at room temperature. Finally 400 μl PBS containing 50 μg/ml propidium iodide was added. % of cells carrying decreased amount of DNA due to apoptosis (sub G₀/G₁ cells) was determined on DNA histograms by flow cytometry using FACScan (BD Biosciences).

2.4. Western blot analysis of Nur77, Bid, Bim and STAT1 expression

Whole cell homogenate was used. 40 μg protein was run on an polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes using the Bio-Rad electrophoresis and transfer system. The free binding sites of membranes were blocked with 5% non fat dry milk powder in 20 mM Tris, 0.1 M NaCl buffer with 0.1% Tween overnight at 4 °C. One μg anti-Nur77 (BD Pharmingen), anti-Bid (R&D Systems), anti-Bim or anti-STAT1 antibodies (Santa Cruz Biotechnology) were added to the membranes. To detect Nur77, Bid, Bim or STAT1 signals peroxidase-labeled anti-mouse IgG (1:1000), anti-rat IgG (1:10,000) or anti-rabbit IgG (1:10,000) were used and the enhanced chemiluminescence was visualized using the ECL System (Amersham). Equal loading of protein was demonstrated with probing the membranes with anti-α-tubulin (Santa Cruz Biotechnology) or β-actin (Sigma-Aldrich) antibodies.

2.5. Affymetrix analysis

Mouse Affymetrix 430Av2 arrays carrying probes for 22690 transcripts were used to identify retinoid-regulated apoptosis genes in mouse thymocytes. Nur77+/+ and −/− control (DMSO exposed) and 4 h 9cRA (0.3 μM) treated thymocyte samples were run in duplicates. Hybridization was carried out in Genomics Core Facility in Heidelberg. Raw intensity data was scaled and normalized in Affymetrix Microarray suite 5.0. Filtering of changed transcripts was carried out in Microsoft Access and Excel software. Increased transcripts were judged as “Present” and increased +2 fold in both duplicates. Decreased transcripts had minimum of −1.6 fold change value in both duplicates.

2.6. qRT-PCR for detecting changes in the mRNA expression of various genes

Thymocytes (5 × 10⁶/ml) were exposed to ATRA or 9cRA for 4 h. At the end of the incubation total RNA was isolated with TRI reagent from treated cells. Transcript quantification was accomplished via quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using Taqman gene expression assay. cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer’s instruction. Real-time monitoring was carried out using an ABI Prism 7900 performing 40 cycles of 94 °C for 12 s and 60 °C for 1 min with FAM-MGB-labeled specific probes (Applied Biosystems). Threshold cycle number was determined using SDS 2.0 software. All samples were assayed in triplicates and the gene expression was normalized to cyclophilin.

2.7. Preparation of mitochondria and Western blot to detect mitochondrial translocation of Nur77/RXR

To obtain mitochondrial fraction of thymocytes, cell homogenates (10⁷ cell/sample) were fractionated by the ProteoExtract Cytosol/Mitochondria Fractionation Kit from Calbiochem according to the manufacturer’s instructions. At the end of the procedure mitochondrial samples were denatured in 5 × Laemmli buffer. Nur77 and RXRα were detected on Western blot by using the antibody against Nur77 (BD Pharmingen) and RXRα (Santa Cruz Biotechnology). The blots were reprobed with antibodies to mitochondrial Hsp60 as loading control or the nuclear Lamin B (Santa Cruz Biotechnology) to detect potential nuclear contamination.

2.8. Intracellular staining for Bcl-2/BH3

To detect intracellular levels of the Bcl-2 BH3 domain thymocytes (2 × 10⁶/sample) were washed twice with PBS, were fixed and permeabilized using 250 μl of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4 °C. After fixation and blocking steps the samples were washed twice using perm/wash solution (200 μl/sample) and stained with the anti-Bcl-2 BH3 domain antibody (Abgent, San Diego, USA 1:100) for overnight at 4 °C. For the detection cells were labeled with FITC- or Cy3-conjugated anti-rabbit IgG and were analyzed by flow cytometry. Data was acquired using FACS Calibur (BD Biosciences) and analyzed using WinMDI 2.9 software.
2.9. Detection of TNF alpha content in thymocyte supernatant

5 × 10^6/0.5 ml wild type thymocytes were plated onto 24-well plates and were stimulated with retinoids and the combination of phosphor dibutyrate and ionomycin for 18 h. The samples were then centrifuged at 4000 g for 5 min. The cytokine quantity of the supernatant culture medium was determined by DuoSet Mouse TNF alpha ELISA Kit (R&D Systems).

2.10. Isolation of CD4^+CD8^+ double positive thymocytes by flow cytometry sorting

Thymocytes isolated freshly from 4 week-old Nur77^+/+ mice were stained in PBS with CD4-PE and CD8-FITC antibodies (Sigma-Aldrich) at room temperature for 10 min. CD4/CD8 double positive thymocytes were separated with FACS Aria III instrument (BD Biosciences). Data analysis was carried out by using BD FACSDiva Version 6.1.3 software.

2.11. Statistical analyses

All the data are representative of at least three independent experiments carried out on three different days. Values are expressed as mean ± S.D. P values were calculated by using two-tailed Student’s t-test for two samples of unequal variance, when data for the wild-type and Nur77 null thymocytes were compared, and Student’s t-test when thymocytes from the same mouse were treated in various ways. Statistical significance is indicated by a single asterisk (P < 0.05).

3. Results

3.1. Nur77 is upregulated and essential in retinoid induced apoptosis of mouse thymocytes

Previous studies in our laboratory have shown that retinoids induce a transcription-dependent apoptosis in mouse thymocytes via activating RARγ [12]. Subsequent studies have revealed that ligation of RARγ can also induce the expression of Nur77 and its target Fasl in a mouse T cell hybridoma cell line [34]. To test whether retinoids could also induce Nur77 in mouse thymocytes, thymocytes isolated from 4 week old mice were exposed to ATRA and 9cRA, and Nur77 expression was detected on both mRNA (Fig. 1A) and protein levels (Fig. 1B). As seen in Fig. 1, both ATRA and 9cRA were able to induce the expression of Nur77 at concentrations where they saturate RARs, however, 9cRA was more effective indicating that ligation of RXR might contribute to the induction of Nur77. To decide whether Nur77 is required for retinoid-induced apoptosis, apoptosis induction by retinoids was tested on thymocytes isolated from both wild-type and Nur77 null mice [32]. As shown in Fig. 1C, retinoids induced a dose dependent cell death in the wild-type thymocytes. In line with the lower expression of Nur77 in the presence of ATRA, ATRA was much less potent in apoptosis induction than 9cRA at each concentrations tested. However, none of these compounds could induce apoptosis in Nur77 null thymocytes indicating an essential role of Nur77 in mediating retinoid-induced apoptosis of thymocytes. Interestingly, even the background rate of apoptosis was lower in Nur77 null thymocytes indicating that the spontaneous cell death involves Nur77-dependent elements. These results were not related to a Nur77-dependent developmental defect of thymocytes, as we did not observe any change in the age-dependent size or thymocyte composition of the Nur77 null thymuses (Fig. 1D and E). Our observation is in line with previously published data [32].

3.2. Fasl, TRAIL, NDG1, GRP65 and Bid are induced by retinoids to initiate thymocyte apoptosis

To identify genes mediating retinoid-induced apoptosis, mRNA levels isolated from control (DMSO-treated) and retinoid-treated thymocytes were compared by Affymetrix 430Av2 arrays carrying probes for 22,690 transcripts. As an effective retinoid, 0.3 μM 9cRA was selected for these studies. We confirmed Nur77, as one of the genes, that was significantly upregulated (Table 1). The Nur77 family consists of Nur77, Nor-1, and Nurr1 with overlapping biological activity, however, only Nur77 was upregulated by 9cRA in mouse thymocytes. These data were also confirmed by qRT-PCR analysis (data not shown). To decide which apoptosis-related genes are regulated in a Nur77-dependent manner during retinoid-induced apoptosis, both wild-type and Nur77 null thymocytes were exposed to 9cRA for 4 h and the changes in their mRNA levels were compared by Affymetrix 430Av2 arrays. We found ten apoptosis related genes to be induced by retinoids (Table 1). Interestingly most of these genes were induced in a Nur77-dependent manner. NDG-1 was already a known Nur77-dependent gene [27]. However G protein-coupled receptor 65 (Gpr65), also known as T-cell death-associated gene 8, which belongs to a group of acid-sensing receptors in the G2A G protein-coupled receptor (GPCR) family coupled to the ade- nylate cyclase [35], and the BH3 interacting domain death agonist (Bid), which is activated by Caspase 8 cleavage and initiates the mitochondrial pathway of apoptosis [36], were unexpected to be found among the Nur77-regulated genes. To confirm the induction of these 3 genes by retinoids, their mRNA expression was also determined by qRT-PCR analysis following 4 h exposure to ATRA or 9cRA. In addition, we also determined the expression of Fasl and TRAIL, which are known Nur77-regulated apoptosis related genes, but were not detectable by the Affymetrix analysis. As shown in Fig. 2, all these genes were induced by retinoids in wild-type thymocytes, but not in Nur77 null thymocytes confirming that these genes are indeed retinoid-induced Nur77 dependent genes. 9cRA was found to be more effective in the induction of all the Nur77-dependent genes than ATRA, in accordance with the higher levels of Nur77 induced by it.

3.3. Caspase 8 activation contributes to the initiation of retinoid-induced apoptosis

Since retinoids induced the expression of TRAIL and Fasl, two cell death receptor ligands, and NDG-1, which similar to death receptors, can also activate Caspase-8 though by unknown mechanisms [27], we decided to check whether Caspase 8 plays a role in retinoid-induced apoptosis. First we tested whether Caspase 8 is cleaved during retinoid-induced apoptosis. As shown in Fig. 3A, cleaved Caspase 8 protein can be detected in retinoid-induced apoptosis. Since Bid was also induced by retinoids (Table 1), and Bid is a Caspase 8 substrate [36], to demonstrate that Caspase 8 is activated during retinoid-induced apoptosis we checked, whether Bid is also cleaved. As shown in Fig. 3B, we could detect cleavage of Bid in thymocytes treated by 9cRA, but not in thymocytes treated by 9cRA in the presence of z-IETD-fmk, a Caspase 8 inhibitor. To test the role of Caspase 8 activation in retinoid-induced apoptosis, we inhibited its activity. Preincubation of thymocytes with z-IETD-fmk significantly attenuated retinoid-induced cell death, but not inhibited it fully (Fig. 3C), similar to the glucocorticoid-induced cell death, in which Caspase 8 has, but not a determining role [37,38]. At the same time z-IETDK completely inhibited Fas-mediated death. These data indicate that Caspase 8 contributes to the initiation of retinoid-induced apoptosis, but the death is not entirely dependent on its activity.

3.4. Caspase 9 also plays a determining role in retinoid-induced apoptosis

Thymocytes are considered to be Type I cells, in which cell death receptor activated Caspase 8 generates sufficient amount of Caspase 3 to drive cell death, without the need for the mitochondrial pathway [39]. Thus Fas mediated apoptosis of thymocytes is not affected by the loss of Caspase 9 [40]. Though we found signs for Caspase 8 activation during retinoid-induced apoptosis, we decided to test by inhibiting Caspase 9, whether retinoid-induced apoptosis requires Caspase 9 activity (thus...
the mitochondrial pathway). Fas-induced apoptosis was used as a control. As seen in Fig. 3C, while the Caspase 9 inhibitor z-LEHD.fmk had only slight effect on the apoptosis induced by the Jo2 anti-Fas antibody, it completely prevented retinoid-induced and dexamethasone-induced apoptosis. These data indicate that though Caspase 8 is activated during retinoid-induced apoptosis, its apoptosis-inducing action requires the mitochondrial pathway of apoptosis involving Caspase 9. In line with this observation we also detected the activation of Caspase 9 (Fig. 3D).

Interestingly, activation of Caspase 8 preceded in time that of Caspase 9 indicating that Caspase 8 plays an initiator role in the retinoid-induced apoptosis.

3.5. Gpr65-mediated signals do not seem to be critical in retinoid-induced apoptosis of thymocytes

 Previous studies have shown that Gpr65 can activate adenylate cyclase [35], and increases in cytosolic cAMP levels might lead to apoptosis in thymocytes by enhancing the production of TNF-α [41] or by inducing Bim [42]. Since 9cRA induced the expression of Gpr65, we decided to test whether addition of retinoids results in TNF-α production by checking its mRNA expression by qRT-PCR and by detecting its protein level by ELISA. Exposure of thymocytes to retinoids did not result in detectable TNF-α production (data not shown). However, as shown in Fig. 4A and B, retinoids significantly induced the expression of Bim at both mRNA and protein levels, but this increase was not inhibited by Rp-cAMPS triethylamine or H89, specific membrane-permeable inhibitors of cAMP dependent protein kinase I and II [43]. Addition of PKA inhibitors had no effect on the retinoid-induced apoptosis of the thymocytes either (data not shown). All together our data indicate that though the expression of Gpr65 is increased, under our culture conditions there is no significant acidification which would activate it and the coupled adenylate cyclase pathway.

3.6. Bim is induced in a STAT1-dependent manner during retinoid-mediated apoptosis of thymocytes

 Previous studies have shown that STAT1 can also regulate Bim expression in thymocytes [44]. The Affymetrix data suggested STAT1 is
also induced during retinoid-induced apoptosis in a Nur77-dependent manner (Table 1), what we could also confirm on protein levels (Fig. 4D). Thus we decided to test by using STAT1 knock out thymocytes [33] whether the induction of Bim expression during retinoid-induced apoptosis is STAT1 dependent. As shown in Fig. 4B and C, our results indicate that enhancement in the Bim expression during retinoid-induced apoptosis is STAT1 dependent. In addition, loss of STAT1, attenuated the retinoid-induced apoptosis of thymocytes (Fig. 4D) indicating that STAT1-induced Bim expression contributes to the initiation of the retinoid-induced apoptosis program.

3.7. Nur77 translocates into the mitochondria during retinoid-induced apoptosis and leads to the exposure of the Bcl-2 BH3 domain

Previous studies have shown that in addition to its transcription-inducing ability, Nur77 can also contribute to apoptosis induction during negative selection in thymocytes by targeting mitochondria, where it converts Bcl-2, an antiapoptotic protein into a proapoptotic molecule after associating with it and leading to the exposure of the Bcl-2/BH3 domain [29]. That is why we tested whether retinoids are capable of inducing the translocation of Nur77 into the mitochondria. As control, we used the phorbol dibutyrate/ionomycin treatment, which mimics the signaling of negative selection. As shown in Fig. 5A, treatment with phorbol dibutyrate/ionomycin resulted in a time dependent translocation of Nur77 into the mitochondria appearing as a wide band due to the heavy phosphorylation of the protein [22]. With much less efficiency, ATRA also induced a time-dependent translocation of Nur77 with a peak at 2 h for each treatment. However, the protein appeared with a much thinner band that was found in phorbol dibutyrate/ionomycin-treated cells, indicating a less prominent or lack apoptosis is STAT1 dependent. In addition, loss of STAT1, attenuated the retinoid-induced apoptosis of thymocytes (Fig. 4D) indicating that STAT1-induced Bim expression contributes to the initiation of the retinoid-induced apoptosis program.

<table>
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<th>Gene description</th>
<th>Symbol</th>
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<td>NDG-1</td>
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<td>G-protein coupled receptor 65</td>
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<td>Myeloid differentiation primary response</td>
<td>Myd116</td>
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*Wild-type and Nur77 null thymocytes were exposed to 0.3 μM 9cRA for 4 h and the changes in their mRNA levels were compared by Affymetrix 430Av2 arrays. Data represent fold changes as compared to the DMSO-treated controls based on two independent experiments. Bold letters highlight dose genes the expression of which is independent of Nur77.*

![Fig. 2](image-url)
Fig. 3. Activation of both Caspase 8 and 9 is required for retinoid-induced apoptosis. (A) Retinoids induce the activation of caspase 8 during apoptosis. Thymocytes were exposed to 0.3 μM 9cRA or 3 μM ATRA, and the cleavage of Caspase 8 was followed in the absence or the presence of 40 μM specific Caspase 8 inhibitor, zIETD-fmk by Western blot at the indicated time points. Tubulin was used as loading control. (B) Bid is cleaved during retinoid-induced apoptosis. Thymocytes were treated with 0.3 μM 9cRA in the absence or the presence of 40 μM zIETD-fmk for 2, 4, and 6 h. Bid expression was determined by Western blot from total cell lysate. β-actin was used as loading control. (C) Retinoids induce the activation of Caspase 9 during apoptosis. Thymocytes were exposed to 0.3 μM 9cRA, 3 μM ATRA or 0.1 μM dexamethasone acetate and the time-dependent cleavage of Caspase 9 was detected by Western blot analysis. (D) Inhibition of both Caspase 8 and 9 prevents retinoid-induced apoptosis. Thymocytes were exposed to 3 μM ATRA, 0.9 μM 9cRA for 12 h, to 0.1 μM dexamethasone acetate or 1 μg/ml Jo2 antibody for 6 h in the presence or absence of 40 μM zIETD-fmk, or 75 μM Caspase 9 inhibitor Z-LEHD-FMK. The percentage of thymocytes in subG1 population was determined in propidium iodide stained samples. Data represent mean ± S.D. of three independent experiments. *Significantly different from the sample, where no caspase inhibitor was present (p < 0.05).

of phosphorylation of it during retinoid-induced signaling. Nur77 can form heterodimers with the retinoid X receptor (RXR), and previous studies indicated that heterodimer formation is required for the nuclear export of Nur77 [31]. Interestingly, we detected mitochondrial translocation of RXR in both Nur77 +/+ and −/− thymocytes. In phorbol dibutyrate/ionomycin-treated cells the kinetics of the mitochondrial appearance of RXR was different from that of Nur77, and the presence or absence of Nur77 did not alter it. In retinoid treated cells, however, the peak of both Nur77 and RXR translocations was detected at 2 h after treatment. Next we checked whether association of Nur77 with mitochondria leads to Bcl-2 conformational change and exposure of its BH3 domain. The Bcl-2 BH3 domain is usually buried within the folded Bcl-2 protein and is undetectable by the Bcl-2/BH3-specific antibodies [29]. Exposure of this domain correlates with the proapoptotic activities of Bcl-2 [29] and can be detected by mimicking signals of negative selection by adding phorbol dibutyrate/ionomycin to the thymocytes [30]. That is why we measured Bcl-2/BH3 exposure during retinoid-induced apoptosis as well, and found a time dependent exposure of the BH3 domain (Fig. 5B). The exposure of the domain was related to Nur77, as the exposure of this domain was not observed in Nur77 null thymocytes exposed to 9cRA.

3.8. Other molecules that might contribute to retinoid-induced apoptosis of thymocytes

In line with our previously published data [9] we detected the induction of transglutaminase 2 upon exposure to 9cRA in a Nur77 independent manner. Transglutaminase 2 is a multifunctional protein crosslinking enzyme, which was shown to be related to the mitochondrial pathway of apoptosis [45,46]. In addition, we found increased expression of two proteins that negatively regulate protein phosphorylation, Gadd34 and Protein tyrosine phosphatase, non-receptor type 6 (Ptpn6). Gadd34 (also known as MyD116) was originally described as a growth arrest and DNA damage-inducible gene. It facilitates protein phosphatase type 1 activity through both direct binding to the protein, as well as through binding to other proteins that also modulate phosphatase activity. Increased expression of Gadd34 was subsequently found to correlate with apoptosis, and forced overexpression of the protein leads to apoptosis [47]. Ptpn6, on the other hand, is known to be required for receptor-mediated cytotoxic signaling that causes intracellular acidification and apoptosis [48]. While the retinoid-induced induction Ptpn6 was Nur77 dependent, the induction of Gadd34 was not.

3.9. Retinoids induce both Nur77-dependent transcription and the appearance of Bcl-2/BH3 domain in the DP thymocytes

Previous studies have shown that retinoids induce apoptosis primarily in double positive (DP) thymocytes [12], but the basal expression of Nur77 shows a differentiation-dependence [49]. While in DP thymocytes the expression of Nur77 is low, both positive and negative selection induces the expression of Nur77, the expression correlating with the strength of the TCR signal. As a result, in the thymus of a transgenic mouse which expressed GFP under the control of the Nur77 promoter, mostly medullary (positively selected) thymocytes were GFP-positive [50]. Thus we decided to test whether the Nur77-dependent events that we observed in non-separated thymocytes are related to DP thymocytes using DP thymocytes received by sorting (Fig. 6A). As shown in Fig. 6B, DP thymocytes expressed Nur77 and reacted to both 9cRA and the PdBu/ionomycin treatment by upregulating Nur77. Since we could generate small amounts of sorted cells we decided to check the retinoid-induction of three Nur77-regulated genes: Gpr65, NDG-1 and Bid. As shown in Fig. 6C, DP thymocytes were exposed to retinoids and then

of phosphorylation of it during retinoid-induced signaling. Nur77 can form heterodimers with the retinoid X receptor (RXR), and previous studies indicated that heterodimer formation is required for the nuclear export of Nur77 [31]. Interestingly, we detected mitochondrial translocation of RXR in both Nur77 +/+ and −/− thymocytes. In phorbol dibutyrate/ionomycin-treated cells the kinetics of the mitochondrial appearance of RXR was different from that of Nur77, and the presence or absence of Nur77 did not alter it. In retinoid treated cells, however, the peak of both Nur77 and RXR translocations was detected at 2 h after treatment. Next we checked whether association of Nur77 with mitochondria leads to Bcl-2 conformational change and exposure of its BH3 domain. The Bcl-2 BH3 domain is usually buried within the folded Bcl-2 protein and is undetectable by the Bcl-2/BH3-specific antibodies [29]. Exposure of this domain correlates with the proapoptotic activities of Bcl-2 [29] and can be detected by mimicking signals of negative selection by adding phorbol dibutyrate/ionomycin to the thymocytes [30]. That is why we measured Bcl-2/BH3 exposure during retinoid-induced apoptosis as well, and found a time dependent exposure of the BH3 domain (Fig. 5B). The exposure of the domain was related to Nur77, as the exposure of this domain was not observed in Nur77 null thymocytes exposed to 9cRA.

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labeled with anti-CD8 and anti-Bcl-2/BH3 antibodies. As shown in Fig. 6D, we could detect the exposure of Bcl-2/BH3 domain in the CD8+ population. Since the majority of CD8+ thymocytes are DP thymocytes, these data indicate that Nur77 can translocate into the mitochondria in these cells. Finally we tested whether the retinoid-induced death of the sorted DP thymocytes is dependent on Caspase 8 and Caspase 9. As shown in Fig. 6E, both inhibitors attenuated retinoid-induced apoptosis in thymocytes. Wild type and STAT1 null thymocytes were exposed to 3 μM ATRA, 0.9 μM 9cRA for 6 h. The percentage of thymocytes in subG1 population was determined in propidium iodide stained samples. Data represent mean ± S.D. of three independent experiments. *Significantly different from the DMSO-treated control (p < 0.05).

4. Discussion

Previous studies in our and other laboratories have shown that retinoids applied alone are capable of inducing apoptosis in mouse thymocytes [11,12] and in T cell lines [34,50]. The studies carried out on the Jurkat T cell line revealed that similar to T cell receptor signaling retinoids can induce the expression of Nur77 [50].

Our data presented in this paper demonstrate that retinoids are capable of inducing the expression of Nur77 also in mouse thymocytes. Retinoids not only induced the expression of Nur77, but retinoid-induced apoptosis was fully dependent on Nur77. This was a surprise, since TCR-mediated apoptosis, which is also coupled to an induction in the expression of Nur77, was not affected by the loss of Nur77 [32]. This observation was explained by the simultaneous induction of Nor1 by TCR signals, which shows overlapping biological activities with Nur77 [51]. However, unlike during TCR signaling, in retinoid-induced apoptosis other members of the Nur77 family were not induced. These data indicate that though Nur77 and Nor1 show overlapping biological activities, they might be regulated by divergent signals.

Our data also demonstrate that 9cRA is more effective in inducing Nur77 than ATRA indicating that ligation of RXR might contribute to the Nur77 induction in mouse thymocytes. Our previous studies have already shown that 9cRA is more effective in inducing apoptosis in thymocytes than ATRA is, but the molecular mechanism was not known. Here we propose that 9cRA is more effective in inducing Nur77, and more Nur77 leads to a more effective apoptosis induction in thymocytes.

Nur77 is a known transcription factor. In a search for the retinoid-induced apoptosis genes, we found the induction of nine apoptosis-related genes, the majority of which was induced in a Nur77-dependent manner. FasL, TRAIL and NDG-1 were previously known to be Nur77 targets in thymocytes [27]. In the present study we identified...
four additional Nur77-dependent genes, Bid, a BH3-only protein, Gpr65, a pH sensitive receptor, Bim, another BH3 only protein, and Ptpn6, a protein phosphatase, during retinoid-induced apoptosis. We have demonstrated that Caspase 8 is activated and Bid is cleaved during retinoid-induced apoptosis.

Since FasL, TRAIL and NDG-1 all could contribute to Caspase 8 activation, we tested the role of Caspase 8 in retinoid-induced apoptosis by a specific Caspase 8 inhibitor, in a concentration at which it only slightly affected dexamethasone-induced cell death. We have demonstrated that Caspase 8 is activated and Bid is cleaved during retinoid-induced apoptosis.

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that retinoids induce a Nur77-dependent cell death program in mouse DP thymocytes, which involves already known and new Nur77-dependent elements, which all together contribute to the induction of the mitochondrial pathway of apoptosis (Fig. 7). Can developing thymocytes be exposed to retinoids under in vivo conditions in the thymus? Data coming from our laboratory and that of Agace indicate that cells located in the thymus are capable of producing RAs generating an endogenous RA milieu within the tissue [6–8]. Thus, we detected an age-dependent mRNA expression of retinaldehyde dehydrogenases (RALDH1 and 2), cellular RA binding protein-II and CYP26A1, proteins responsible for the synthesis, nuclear transport and degradation of RA in the postnatally developing thymus [6]. We could also demonstrate the existence of an age- and RALDH-dependent production of RAR-activating ligand(s) by measuring the induction of an RAR-responsive transgene in two independent transgenic mouse strains, in which the expression of the transgene reflects functionally relevant retinoic acid synthesis [6]. We also found that their production is related to engulfing macrophages [9]. Surprisingly, however, we could not detect the presence of any of the classical retinoids in the mouse thymus [6], instead metabolites of the retinol saturase pathway seem be produced [53]. Thus, our data indicate that in vivo novel retinoids might contribute to the regulation of thymocyte apoptosis which are produced in the thymic cortex during the course of the constant apoptotic cell engulfment.

5. Conclusions

Our data presented in this paper indicate that retinoids induce a Nur77-dependent apoptosis in immature thymocytes. Data coming from our laboratory and that of Agace indicate that cells located in the thymus are capable of producing RAs generating an endogenous RA milieu within the tissue [5–9], and the source of retinoids in the postnatal thymus are the engulfing macrophages [9]. Besides retinoids, various other molecules, such as TGF-β, carbon monoxide, ATP or adenosine, are also released by macrophages while they engulf apoptotic cells. Interestingly, all these molecules have been described to induce or promote apoptosis in thymocytes in the absence of TCR signaling. Thus we propose that thymic macrophages, because they continually engulf apoptotic cells, might constantly provide these cell death-inducing signals including retinoids and thus contribute to the formation of a thymic milieu that ensures the effective induction of “death by neglect” [54].
Fig. 7. Proposed model for retinoid-induced apoptosis in DP mouse thymocytes. Retinoids induce the expression of Nur77. The transcription factor Nur77 induces the expression of Fasl, TRAIL, NGD-1, Gpr65 and STAT1. Fasl, TRAIL and NGD-1 may contribute to Caspase 8 activation. Caspase 8 will cleave Bid, the expression of which is also enhanced during retinoid-induced apoptosis. Retinoid-induced STAT1 leads to the upregulation of Bim. Retinoids also induce the expression of transglutaminase 2. Cleaved Bid, TG2 and Nur77 can directly act on the mitochondria to initiate the mitochondrial pathway of apoptosis.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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