Different Ways to Induce Apoptosis by Fenretinide and All-trans-retinoic Acid in Human B Lymphoma Cells

GABOR BARNA1, ANNA SEBESTYÉN1, SILKE WEISCHEDE1, ISTVÁN PETAK1, RUDOLF MIHALIK2, FRANCA FORMELLI3 and LASZLO KOPPER1,2

1First Department of Pathology and Experimental Cancer Research, Faculty of Medicine, Semmelweis University, Budapest;
2Molecular Pathology Research Group, Joint Organization of Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary
3Istituto Nazionale Tumori, Chemoprevention Unit, Milan, Italy

Abstract. All-trans-retinoic acid (ATRA) and its synthetic analog fenretinide (4HPR) are potent anticancer drugs. Only a few reports are available about the effects of retinoids on B lymphoma cells. In our study, non-Hodgkin lymphoma cells (HT58) were treated with ATRA and 4HPR. Both agents induced cell death time- and dose-dependently. Reactive oxygen species (ROS) production was elevated in 4HPR-treated cells, but not in ATRA-treated cells. The depolarization of the mitochondrial membrane occurred earlier after ATRA than after 4HPR treatment. Z-VAD-fmk, the general caspase inhibitor, decreased the DNA fragmentation in ATRA-treated cells, but simultaneously increased necrosis. However, z-VAD-fmk did not influence the DNA fragmentation in 4HPR-treated cells. Endonuclease G was released from the mitochondria during 4HPR treatment, which could be an inducer for caspase-independent DNA fragmentation. Our results suggest that natural (ATRA) and synthetic (4HPR) retinoids induce different apoptotic pathways in B lymphoma cells, which is particularly relevant for their potential use in leukemia treatment.

Retinoids are natural and synthetic analogs of vitamin A, involved in the control of cell differentiation and proliferation. All-trans-retinoic acid (ATRA) and its synthetic analog, N-(4-hydroxyphenil)-retinamide (fenretinide, 4HPR), are potent anticancer drugs. They are currently employed in the treatment of acute promyelocytic leukemia (1) and neuroblastoma (2), and are in clinical trials for the prevention of ovarian (3), lung (4, 5) and breast tumors (6).

These agents are antiproliferative as well as apoptotic in in vivo (7, 8) and in vitro models, including breast cancer cells (9), ovarian carcinoma cells (10), acute lymphoblastic leukemia cells (11) and neuroblastoma cells (12). Only a few reports are available on the effects of retinoids on B lymphoma cells.

The apoptotic program can be activated either by the death-receptor (extrinsic) or, similar to many chemotherapeutic agents, by the mitochondrial (intrinsic) apoptotic pathway. Retinoids are thought to function via their receptors (RAR, RXR) or without receptors (13). Both ATRA and 4HPR induced depolarization of mitochondria, but in different ways (14, 15). They were able to regulate the expression of the Bcl-2 family members (15). In certain cell types, ATRA binds to adenine nucleotide translocate (ANT) and induces the opening of mitochondrial permeability pores (MPP) (16), while, in other cell types, 4HPR modulates the respiratory chain and increases reactive oxygen species (ROS) production, thus producing the same effect (17). After these events, the caspase cascade is activated and DNA fragmentation is induced.

We compared the mechanism of the apoptotic effect of ATRA and 4HPR in a non-Hodgkin lymphoma cell line of B-cell origin.

Materials and Methods

Cell culture and treatment. Experiments were performed on a human non-Hodgkin lymphoma cell line of B-cell origin (HT58) (18). The cells were cultured in RPMI-1640 (Sigma, St. Louis, MI, USA), with 10% fetal calf serum (FCS), glutamine and gentamycin. For all experiments, cells growing in the exponential phase of the culture were used and they were cultured in RPMI-1640 with 3% FCS, because a high FCS concentration inhibits entry of the retinoids into the cells (19). The cells were treated in 24-well plates at a density of 2×10⁵ cells/ml/well.

The cells were treated with ATRA 20-40 μM (Sigma) or 4HPR 3-10 μM (kindly provided by R.W. Johnson Pharmaceutical Research Institute, Spring House, PA, USA) for different times up to 48 h. The inhibitors of caspase-8, z-IETD-fmk and caspase-9,
Figure 1. ATRA and 4HPR induced cell death time- and dose-dependently in HT58 lymphoma cells. HT58 cells were incubated with different concentrations of the two retinoids for 24 hours (A, B).

Figure 2. Effect of the general caspase inhibitor z-VAD-fmk (100 μM) on 4HPR- and ATRA-induced cell death at 24 hours. DNA fragmentation and necrosis were measured simultaneously, as described in Materials and Methods. The empty bars show the proportion of cells with fragmented DNA; black bars show the necrotic cells. An empty and a gray bar display the total cell death. (A) z-VAD-fmk did not affect the DNA fragmentation induced by 4HPR. (B) z-VAD-fmk decreased DNA fragmentation, but did not decrease cell death in ATRA-treated cells, because the necrotic population was increased.

Figure 3. ATRA- and 4HPR-induced depolarization of the mitochondrial membrane. (A) ATRA and 4HPR induced depolarization of the mitochondrial membrane but by different modes. (B) The process induced by ATRA was inhibited by z-VAD-fmk (100 μM).
z-LEHD-fmk (both from Pharmingen Becton-Dickinson Co., San Diego, CA, USA) were used at 50 μM; and the general caspase inhibitor z-VAD-fmk (20) at 100 μM. The cells were treated with the inhibitors 0.5 h before retinoid treatment.

Apoptosis detection. Apoptotic cells were detected according to the methods described by Gong et al. (21). The cells were fixed in ethanol (–20°C, 70%), followed by alkaline extraction (200 mM Na₂HPO₄, pH 7.8) and ethidium bromide staining. Measurements were made on a FACScan flow cytometer (Becton-Dickinson Co., San Diego, CA, USA) and the data were analyzed by Winlist software (Verity Software House, Topsham, ME, USA).

DNA fragmentation and necrosis detection. DNA fragmentation and necrosis were detected according to the method described by Imre et al. (22). The treated cells were incubated with propidium iodide at a final concentration of 5 μg/ml for 15 min, then they were fixed in ethanol and prepared for flow cytometric measurements. Necrotic but not DNA-fragmented cells have decreased forward scatter (FSC) / side scatter (SSC) and they can be distinguished from living cells.

Figure 4. ATRA- and 4HPR-induced translocation of BAX from the cytosol to the mitochondria. (A) control cells; (B) 4HPR treatment (8 μM, 6 h); (C) ATRA treatment (40 μ, 6 h); (D) 4HPR treatment (16 h); (E) ATRA treatment (16 h). Mitochondria were labelled with CMTMRos (50 nM). The BAX protein was labelled with anti-human BAX antibody and the translocation was detected by confocal microscopy.
Depolarization of mitochondria. ATRA- and 4HPR-treated cells were incubated with DiOC6 (final concentration: 10 nM) (Molecular Probes, Eugene, OR, USA) and propidium iodide (final concentration: 5 µg/ml) (Sigma) for 15 min before flow cytometric measurements.

Measurement of intracellular ROS. For measuring intracellular ROS, an oxidation-sensitive probe, H$_2$DCF-DA (Molecular Probes), was used. Cells were incubated with the dye (final concentration: 50 µM) for 15 min before retinoid treatment. Then fluorescence was measured by flow cytometry.

Detection of BAX, Endonuclease G and AIF translocation. The cells for the detection of the translocation of proteins were prepared according to the method of Barna et al. (23). Specific polyclonal antibodies for BAX (1:20) (DAKO, Glostrup, Denmark), Endonuclease G (Endo G) (1:30) (Sigma) and apoptosis-inducing factor (AIF) (1:30) (MBL, Naka-Ku, Nagoya, Japan) were used. The samples were studied by confocal microscope (Biorad Labs, Hercules, CA, USA, MRC1024).

Results

ATRA and 4HPR induced apoptosis time- and dose-dependently. Both drugs induced apoptosis time- and dose-dependently (Figure 1A, B). Apoptosis was induced by ATRA rather rapidly, reaching an early plateau (at about 6 h using 30 µM), whereas the effect of 4HPR developed more slowly. 4HPR was more potent than ATRA because it induced apoptosis at 5-10 µM (Figure 1A), while higher concentrations of ATRA (30-40 µM) were needed to produce the same effect (Figure 2B). In the following experiments, equipotent doses of ATRA and 4HPR were used.

Effect of caspase inhibitors. The role of caspases in DNA fragmentation was estimated by different inhibitors of caspases (z-IETD-fmk, z-LEHD-fmk, z-VAD-fmk). All the inhibitors decreased the DNA fragmentation in ATRA-treated cells. At 24 h, the general caspase inhibitor, z-VAD-fmk, was the most effective in inhibiting DNA fragmentation induced by ATRA, while at 48 h the caspase-8 inhibitors z-IETD-fmk and z-VAD-fmk were more effective than the caspase inhibitor z-LEHD-fmk. z-VAD-fmk decreased DNA fragmentation in ATRA-treated cells, however, at the same time the proportion of necrotic cells was increased. In z-VAD-fmk-pretreated cells, DNA fragmentation was reduced from 60% to 30%, but the necrosis increased from 5% to 35%. z-VAD-fmk only slightly (from 85% to 75%) reduced DNA fragmentation induced by the two lowest tested doses of 4HPR. For both agents, the total amount of dead cells (apoptotic + necrotic) was not effected by the addition of z-VAD-fmk (Figure 2A, B).

ATRA and 4HPR induced mitochondrial depolarization. Both retinoids induced the depolarization of the inner mitochondrial membrane, which is an important step in death receptor-independent apoptosis. Depolarization of the mitochondria started at 2 h after ATRA treatment and increased up to 4 h (Figure 3A). The effect of 4HPR, at 5 times lower concentrations, started later (6 h), but reached the same level as ATRA. In ATRA-treated cells, z-VAD-fmk, which decreased the DNA fragmentation, also decreased the depolarization of mitochondria (Figure 3B). The effect of z-VAD-fmk on 4HPR-induced depolarization was not studied, because no effect had been observed on DNA fragmentation.

ATRA and 4HPR induced the translocation of BAX. One common consequence of mitochondrial depolarization is the activation of pro-apoptotic and/or inhibition of anti-apoptotic members of the Bcl-2 family. Both retinoids decreased the expression of Bcl-2 protein, but the expression of BAX protein did not change (data not shown). The function of BAX is mainly dependent on its translocation from the nucleus to the mitochondria. Confocal microscopy showed that ATRA induced translocation of BAX in 30% of the cells at 6 h after the treatment, while the action of 4HPR was much less (it appeared in 10% of cells at 16 h) (Figure 4).
4HPR induced reactive oxygen species. Besides the pro-apoptotic members of the Bcl-2 protein family, ROS activated mitochondrial permeability. An increase in ROS production was detected 15 min after 4HPR treatment and continued to increase with concentration and time (Figure 5A). ATRA did not change ROS production (Figure 5B).

ATRA and 4HPR induced Endonuclease G release from mitochondria. z-VAD-fmk did not inhibit DNA fragmentation induced by 4HPR, therefore we looked for changes in the localization of two caspase-independent molecules. In control cells, Endo G and AIF were colocalized with CMTMRos chloromethyl-tetramethyl-rosamin [Mitotracker Orange (Molecular Probes)] labelled mitochondria. Both 4HPR and ATRA induced an even intracellular spreading of Endo G as a sign of release from the mitochondria. This effect appeared earlier after 4HPR (at 6 h) than after ATRA treatment (at 12 h) (Figure 6). AIF remained dotted after both drugs.

Discussion

In this study, we compared the apoptotic effect of a natural retinoid (ATRA) and its synthetic analog (4HPR) in non-Hodgkin lymphoma cells of B-cell origin. ATRA was used at retinoid (ATRA) and its synthetic analog (4HPR) in non-Hodgkin lymphoma cells of B-cell origin. ATRA was used at high concentrations (higher than clinically equivalent) because it was less potent (~5 fold) than 4HPR in inducing apoptosis.

Both agents induced apoptosis using the intrinsic apoptotic pathway in HT58 cells, as previously shown in HL60 promyelocytic leukemia cells (24). We demonstrated here that the key event is the depolarization of mitochondria.

The depolarization induced by ATRA was an early reaction. It began at 2 h after treatment and was inhibited by z-VAD-fmk. These results indicate the role of mitochondria in apoptosis induction. Similar results were found by Chen et al. (25) in multiple myeloma cells, where mitochondria and caspases worked in a positive feedback loop. An early small cytochrome c release can activate caspases, contributing to the depolarization of other mitochondria in the cells. It is possible that this sequence is the consequence of the translocation of BAX (from the cytosol to the mitochondria) which is helped by a cleaved fragment of BID (23, 26). The cleavage of BID could be performed by caspase-8 (27), by catepsin-D (28) or by calpain (29).

In HT58 cells, the inhibition of caspase-8 had little effect on apoptosis induced by the two retinoids, suggesting the role of other proteases in the amplification loop. z-VAD-fmk decreased DNA fragmentation in ATRA-treated cells at 24 h, but increased plasma membrane permeability, which is an early sign of necrosis. This suggests that a pathway other than caspase-dependent apoptosis is accessible and could induce cell death. This process has been shown to be initiated by disruption of the mitochondria and ATP depletion, leading to stress (30). Again, not only caspases, but other proteases (e.g. calpain) (31) could also contribute to necrosis.

4HPR elevated the ROS level in HT58 cells, as in other cell types (23), and induced apoptosis via the mitochondria. The DNA fragmentation caused by 4HPR was not prevented by z-VAD-fmk. In another lymphoma cell line, the apoptosis induced by 4HPR was lowered by z-VAD-fmk, suggesting caspase dependency (32). It is highly possible that the apoptosis machinery works differently in different tumors, even in closely related cell lines. This underlines the importance of identifying the key steps in the apoptotic pathway in order to apply the relevant pro- or anti-apoptotic agent.

It is known that caspase-independent apoptosis can be elicited by different factors, such as AIF and Endo G (33). Both factors can be released from mitochondria together with cytochrome c, and cause DNA fragmentation without caspases (34, 35). Endo G was released from the mitochondria after 4HPR treatment. This may result in the failure of z-VAD-fmk to inhibit DNA fragmentation. (In fact, some Endo G escaped after ATRA treatment as well, but it happened at a later stage, when the level of apoptosis had reached a plateau.)

These results help in understanding the effects of the two retinoids with a view to developing new therapies. 4HPR can be used in tumors, e.g. in non-Hodgkin lymphomas where the caspase activity is low or missing due to overexpression of IAP family members (36).

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References

Figure 6. Release of Endonuclease G from mitochondria by 4HPR. Confocal microscopy was used to detect Endonuclease G release from the mitochondria. (A) control cells; (B) 4HPR treatment (8 μM, 6 h); (C) ATRA treatment (40 μM, 6 h); (D) 4HPR treatment (12 h); (E) ATRA treatment (12 h). Mitochondria were labelled with CMTMRos and proteins by specific antibody. The cells are shown by phase contrast.


