Induction of apoptosis by the novel retinoid AHPN in human T-cell lymphoma cells involves caspase-dependent and independent pathways

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Abstract

Retinoids play an important role in the control of lymphocyte function and homeostasis in the thymus. In this study, we show that the induction of growth arrest and apoptosis in a variety of T-cell lymphoma cell lines, including Jurkat and Molt-4 cells, is highly specific for the synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) since all-trans retinoic acid (RA), the RAR-selective retinoid TTAB, the RXR-selective retinoid SR11217 and the retinoid SR11302 exhibiting selective anti-AP1 activity, do not induce apoptosis or cause growth arrest. These findings support the concept that the effects of AHPN on proliferation and induction of apoptosis are mediated by a novel signaling pathway. AHPN-induced apoptosis is associated with an induction of internucleosomal DNA-fragmentation, increased annexin V binding and a 30-fold stimulation of caspase-3-like activity. Overexpression of Bcl-2 in Molt-4 cells greatly inhibits the induction of apoptosis by AHPN as indicated by the inhibition of DNA-fragmentation, annexin V binding and caspase-3-like activity. However, Bcl-2 overexpression does not interfere with the ability of AHPN to cause growth arrest or accumulation of cells in the early S-phase of the cell cycle, indicating that the effects of AHPN on growth arrest can be uncoupled from the effects on apoptosis. The caspase inhibitor Z-VAD-FMK, at concentrations that totally block caspase activity, delays but does not prevent cell death and does not affect the accumulation of cells in the S-phase of the cell cycle. Our results show that induction of caspase-3-like activity plays an important role in the execution of AHPN-induced apoptosis but cells can undergo cell death in the absence of this activity suggesting that AHPN-induced cell death involves caspase-dependent and -independent mechanisms.

Keywords: retinoids; apoptosis; lymphoma; caspase; cell proliferation

Abbreviations: AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; PARP, poly(ADP-ribose)polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RA, retinoic acid

Introduction

Apoptosis plays an important role in morphogenesis during normal embryonic development, homeostasis in many tissues in the adult and in the downmodulation of immune responses (Webb *et al*, 1997; Mountz *et al*, 1996; Jacobson *et al*, 1997). Defects in the control of apoptosis have been linked to numerous pathological processes, including cancer, auto-immune and neurodegenerative diseases (Thompson, 1995; Jacobson *et al*, 1997; Webb *et al*, 1997).

Programmed cell death is a multi-step process and an increasing number of genes have been identified that are involved in either the control or execution of apoptosis (Boise *et al*, 1995; Korsmeyer, 1995; Kumar and Flavin, 1996; Kroemer, 1997). One set of genes involved in cell death encode structurally-related cysteine proteases, named caspases, which cleave specific Asp-X peptide bonds (Nicholson, 1994; Fernandez-Alnemri *et al*, 1994; Kumar and Flavin, 1996). All known members of this family are synthesized as single-chain proenzymes which require proteolytic processing to produce active, heterodimeric proteases. Activation of caspases plays a critical role in the execution of apoptosis in T-lymphocytes and T-cell lymphomas as well as other cell types (Enari *et al*, 1996; Armstrong *et al*, 1996; Longthorne and Williams, 1997). Members of the Bcl-2 gene family encode another set of proteins that regulate apoptosis in either a positive or negative manner (Boise *et al*, 1995; Kroemer, 1997; Reed, 1997). Bcl-2 has been shown to inhibit the activation of caspases and protect T cells from undergoing apoptosis in response to a variety of signals (Hockenbery *et al*, 1990; Miyashita and Reed, 1992; Zhang *et al*, 1996; Kroemer, 1997). Recent studies have indicated that the suppression of caspase activation by Bcl-2 is related to inhibition of the release of cytochrome c from mitochondria (Kluck *et al*, 1997; Yang *et al*, 1997). However, cytochrome c-independent mechanisms of caspase activation have also been described (Chauhan *et al*, 1997).

T-lymphocytes have been used extensively as a model system to study apoptosis (Ucker, 1987; Smith *et al*, 1989; Mountz *et al*, 1996; Longthorne and Williams, 1997). Negative selection is a normal process in the thymus by

which self-reactive T cells undergo apoptosis after the interaction of the self-antigen with the T cell receptor and apoptosis can be induced by a number of other stimuli, including glucocorticoids, ceramide, irradiation, tumor necrosis factor, Fas ligand and anti-cancer drugs (Smith *et al*, 1989; Miyashita and Reed, 1992; Mountz *et al*, 1996; Zhang *et al*, 1996; Longthorne and Williams, 1997). Retinoids play also an important role in the control of homeostasis in the thymus and of lymphocyte function, and can effect proliferation and apoptosis in lymphocytes and lymphoma cells in either a positive or negative manner (Iwata *et al*, 1992; Vacchio and Ashwell, 1993; Yang *et al*, 1995a,b; O'Connell *et al*, 1996; Piedrafita and Pfahl, 1997; Szondy *et al*, 1997; Zhang and Jetten, 1997; Lomo *et al*, 1998). Many of the biological and molecular effects of retinoids are mediated by nuclear retinoid receptors (Giguère, 1994). The inhibition of activation-induced apoptosis by retinoids is due to an inhibition of Fas ligand mRNA synthesis and requires both RAR and RXR activation (Yang *et al*, 1995b; Bissonnette *et al*, 1995). In contrast, the inhibition of cell death in

lymphocytes by 14-hydroxy-4,14-retroretinol is mediated through a mechanism not involving nuclear retinoid receptors (O'Connell *et al*, 1996). The inhibition of proliferation and induction of apoptosis in a variety of carcinoma cell lines by the synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) also occurs through a novel mechanism (Shao *et al*, 1995; Schadendorf *et al*, 1996; Oridate *et al*, 1997; Sun *et al*, 1997; Piedrafita and Pfahl, 1997; Hsu *et al*, 1997; Adachi *et al*, 1998).

T cell lymphocytes and lymphomas are excellent *in vitro* cell systems to study the signaling pathway by which AHPN induces apoptosis since the regulation of apoptosis in lymphocytes is well-studied and because of the important regulatory roles that various retinoids play in lymphocyte function. In this study, we demonstrate that the induction of growth arrest and apoptosis in a variety of T cell lymphoma cells, including Jurkat and Molt-4 cells, is highly specific for AHPN since all-*trans* retinoic acid, an RAR-, an RXR-, and an anti-AP-1-selective retinoid were unable to induce apoptosis or cause accumulation of cells in the early S-

Figure 1 Effect of AHPN and several other retinoids on the proliferation of Jurkat, Molt-4 and S49-neo lymphoma cells and the T-cell hybridoma DO11.10. (A) Comparison of effects of various retinoids on the growth of Molt-4 and Jurkat cells. Cells were treated with 1 μM of the indicated retinoid for 32 (Molt-4; hatched bars) or 24 h (Jurkat; open bars). Cell survival was determined with the MTT assay. The results shown are representative of two independent experiments. (B) Cells (10^5 cells/well) were treated with AHPN at the concentrations indicated. After 4 days of incubation cell numbers were determined. □, Jurkat; ●, S49-neo; △, Molt-4; ○, DO11.10 cells. (C). Effect of AHPN on cell survival of Jurkat cells as a function of the period of treatment. Cells were treated with (●) and without (○) 1 μM AHPN and at the times indicated cells were stained with Trypan blue. The number of cells excluding Trypan blue (cell survival) was determined and plotted. The difference between duplicate cell numbers in B and C varied by no more than 5%. Control cells received vehicle (0.1% DMSO) only

phase of the cell cycle. AHPN-induced apoptosis in T cell lymphomas is associated with an induction of DNA fragmentation, as indicated by 'ladder' and TUNEL assay, increased annexin V binding and caspase-3-like activity. The induction of caspase activity, annexin V binding and DNA fragmentation by AHPN but not growth arrest was inhibited in cells overexpressing Bcl-2 or cells treated with the caspase inhibitor Z-VAD-FMK. Our results suggest that although activation of caspases accelerate the execution of apoptosis, AHPN-induced cell death can occur independently of caspase activation.

Results

AHPN-specific inhibition of cell proliferation

Previous studies have reported that various retinoids can affect proliferation and apoptosis of lymphocytes and lymphoma cells in multiple ways and can influence these processes in a stimulatory as well as inhibitory manner (Yang *et al*, 1995a,b; Iwata *et al*, 1992; O'Connell *et al*, 1996; Szondy *et al*, 1997; Lomo *et al*, 1998). In this study, we examined the effects of several retinoid receptor-selective retinoids on the growth of the human T cell lymphoma cell lines Jurkat and Molt-4. The results show that the growth-inhibitory effect was highly specific for AHPN since all-*trans* RA, the RAR-selective retinoid TTAB, the RXR-selective retinoid SR11217, and the anti-AP1-selective retinoid SR11302 had little effect on cell growth (Figure 1A). The growth-inhibitory effect could be observed at concentrations as low as 0.1 μ M AHPN and was not limited to Jurkat and Molt-4 cells but could also be demonstrated in rat lymphoma S49 and the mouse T-cell hybridoma DO11.10 cells (Figure 1B). Combined treatment with RXR and RA, TTAB or AHPN did not further increase the effect on cell growth, and in contrast to normal thymocytes (Szondy *et al*, 1997), RAR α -selective retinoids were unable to inhibit AHPN-induced apoptosis (not shown). To determine whether the growth inhibition by AHPN was related only to growth arrest or also involved cell death, cell survival in AHPN-treated Jurkat cells was monitored by a Trypan blue exclusion assay. After a lag of about 8 h, AHPN caused a dramatic decrease in the viability of Jurkat cells indicating that AHPN induced cell death (Figure 1C).

Induction of internucleosomal fragmentation by AHPN

In order to determine whether the AHPN-induced reduction in cell viability in lymphoma cells was related to apoptosis, genomic DNA from AHPN-treated and untreated Jurkat and S49 cells were isolated and examined for degradation into oligonucleosomal fragments by electrophoretic analysis. As shown in Figure 2, DNA from Jurkat and S49 cells treated for 8 h with AHPN displayed an oligonucleosomal pattern of DNA degradation ('ladder') characteristic for apoptosis. DNA from dexamethasone-treated S49 cells was used as positive control for the induction of DNA fragmentation during apoptosis (Figure 2).

We next analyzed DNA fragmentation by the TdT dUTP-FITC nick end-labeling (TUNEL) technique using flow cytometric analysis (Gavrieli *et al*, 1992). These analyses demonstrated that compared to control cells dUTP-FITC labeling was significantly increased in Jurkat cells treated for 8 h with AHPN (Figure 3, left panels). These results indicate that AHPN treatment results in increased formation of double-stranded DNA breaks and support AHPN-induced apoptosis in these cells.

Induction of annexin V binding by AHPN

In many cell types the induction of apoptosis is associated with plasma membrane changes including loss of membrane phospholipid asymmetry which can be analyzed by the translocation of phosphatidylserine from the inner layer of the plasma membrane to the outer layer (Vermes *et al*, 1995). The translocation of phosphatidylserine can be monitored by measuring the binding of FITC-conjugated annexin V, a Ca²⁺-binding protein with high affinity for phosphatidylserine, to cells by flow cytometry. As shown in Figure 3 (right panels), treatment of Jurkat cells with AHPN dramatically increased the number of cells with high level of annexin V-FITC binding. Most of these appeared to represent cells in the intermediate- to late-stage of apoptosis. These findings are consistent with the induction of apoptosis in lymphoma cells.

Figure 2 Oligonucleosomal degradation of genomic DNA in AHPN-treated Jurkat, S49-neo and S49-Bcl-2 cells. Cells were treated with 1 μ M AHPN and after 8 h DNA was isolated and analyzed by 1% agarose gel electrophoresis as described in Materials and Methods. Genomic DNA isolated from S49 cells treated with 0.3 μ M dexamethasone (Dex) for 24 h was included as a positive control. The size of the markers (left lane) is indicated in the left margin

Figure 3 Induction of apoptosis by AHPN in Jurkat cells as indicated by TUNEL and annexin V binding assays. (A, B) Cells were treated with DMSO (control) or $1 \mu\text{M}$ AHPN for 8 h. Cells were then fixed and analyzed for apoptosis with the nick end-labeling technique (TUNEL) by flow cytometry. (C, D) Increased annexin V-FITC binding in AHPN-treated Jurkat cells. Cells were treated with DMSO or $1 \mu\text{M}$ AHPN for 8 h. Cells were then analyzed for annexin V-FITC binding by flow cytometric analysis. (A, C): Control, DMSO-treated cells; (B, D): AHPN-treated cells. Vertical axis indicates relative propidium iodide (PI) staining

Induction of caspase-3-like activity by AHPN

Activation of cysteine proteases is one of the key events in the induction of apoptosis (Nicholson 1994; Fernandes-Alnemri *et al*, 1994; Kumar and Flavin, 1996). To study the role of caspase activation in the AHPN-induced cell death pathway in lymphoma cells, we analyzed the activation of cysteine proteases by AHPN in Jurkat cells by determining the ability of cellular extracts to cleave the fluorogenic substrates YVAD-AFC or DEVD-AFC which are effective substrates for caspase-1-like and caspase-3-like proteases, respectively (Slee *et al*, 1996; Armstrong *et al*, 1996). As shown in Figure 4, extracts from control Jurkat and Molt-4 cells had relatively low proteolytic activity with either YVAD-AFC or DEVD-AFC as substrate. The basal caspase-3-like activity was higher in Molt-4 cells while caspase-1-like activity was higher in Jurkat cells. Extracts from AHPN-treated Jurkat and Molt-4 cells contained, respectively, 135- and 27-fold higher levels of DEVD-AFC cleaving activity than extracts from untreated cells while their ability to cleave YVAD-AFC was not much different from that of untreated cells. These results demonstrate that AHPN treatment dramatically induces caspase-3-like activity and has little effect on caspase-1-like activity in these cells.

The induction of caspase-3-like activity by AHPN occurred in a time-dependent manner (Figure 5A). In Jurkat cells, caspase-3-like activity was induced after a lag-phase of about 4 h. After 8 h the caspase-3-like activity was increased about 50-fold. The induction of caspase activity was also dependent on the concentration of AHPN (Figure 5B). A sharp increase in caspase-3-like activity was observed between 0.25 and $0.5 \mu\text{M}$ AHPN.

To determine the specificity of this induction, we compared the effects of all-*trans* RA, AHPN, the RAR-,

Figure 4 Effect of AHPN on the caspase-1- and caspase-3-like activity in Jurkat (A) and Molt-4 cells (B). Cells were treated with $1 \mu\text{M}$ AHPN (+) or with solvent (0.1% DMSO)(-) for 18 h and then assayed for caspase activity using either YVAD-AFC or DEVD-AFC as substrate as described in Experimental Procedures. The experiment was performed in triplicate. Error bars indicate standard error

Figure 5 Time- and dose-dependent induction of caspase-3-like activity by AHPN in Jurkat cells. (A). Cells were treated with $1 \mu\text{M}$ AHPN and at the indicated times cells were collected, lysed and cellular extracts assayed for caspase-3-like activity using DEVD-AFC as a substrate. The rate of DEVD-AFC cleavage was determined and plotted as pmoles AFC/min/mg protein. (B). Cells were treated with AHPN at the concentrations indicated. After 6 h cells were collected and assayed for caspase-3-like activity

RXR- and anti-AP-1-selective retinoid on the induction of caspase-3-like activity (Figure 6A). Only AHPN was able to induce caspase-3-like activity in Jurkat cells. The specificity with which these retinoids induced caspase activity correlated with their ability to inhibit cell growth (Figure 1A). Previous studies have shown that RAR- and RXR-selective retinoids can inhibit activation-induced apoptosis in T cell hybridomas by inhibiting the synthesis of Fas ligand (Yang *et al*, 1995a,b; Bissonnette *et al*, 1995). Therefore, we examined whether these retinoids could also inhibit the induction of apoptosis by AHPN. As shown in Figure 6B, neither the RXR-selective retinoid nor the RAR-selective retinoid TTAB affected caspase activation by AHPN in either a positive or negative manner. And in

Figure 6 Induction of caspase-3-like activity is specific for AHPN. Jurkat cells were treated with 1 μ M all-*trans* RA, AHPN, an RAR-, RXR-, or an anti-AP-1-selective retinoid (A) or with a combination of AHPN and the RAR- or RXR-selective retinoid (B). After 6 h incubation cells were collected and assayed for caspase-3-like activity. The rate of DEVD-AFC cleavage was determined and plotted

contrast to thymocytes (Szondy *et al*, 1997), RAR α -selective retinoids did not inhibit AHPN-induced apoptosis and simultaneous treatment with RXR- and RAR-selective retinoids did not induce caspase activity (not shown).

Induction of PARP cleavage

Poly(ADP-ribose)polymerase (PARP) is one of several proteins that have been identified to function as a substrate for caspases (Tewari *et al*, 1995; Kumar and Flavin, 1996). DEVD is the site of cleavage of the 113 kD PARP by caspase-3 yielding two fragments of 89 and 24 kD. We examined the ability of cellular extracts from AHPN-treated and untreated Molt-4 cells to cleave a radiolabeled, truncated form of PARP (tPARP). As shown in Figure 7A, extracts from untreated cells contained only the 48 kD tPARP while AHPN-treated cells contained in addition the 28 and 20 kD cleaved products. The generation of these cleaved products was dependent on the time of AHPN-treatment.

Effect of the caspase inhibitor Z-VAD-FMK on cell death

Z-VAD-FMK is a cell-permeable peptide that at high concentrations irreversibly inhibits the activity of many caspases (Slee *et al*, 1996; Stefanis *et al*, 1996; Longthorne and Williams, 1997; Thornberry *et al*, 1997). To determine the effect of caspase inhibition on AHPN-induced cell death, we examined the relationship between the effect of increasing concentrations of Z-VAD-FMK on caspase activity and cell death in Molt-4 cells. Z-VAD-FMK completely inhibited AHPN-induced caspase-3-like activity (Figure 8A) and caspase-1-like activity (not shown) and also greatly reduced DNA fragmentation in AHPN-treated cells as measured by TUNEL analysis (Figure 8B). Despite the dramatic reduction in caspase activity and DNA-fragmentation, Z-VAD-FMK decreased but did not prevent AHPN-induced cell death (Figure 8A). Similar observations have been reported for the action of Z-VAD-FMK on cell death induced by oncogenes, DNA

A. MOLT-4

B. MOLT-bcl2

Figure 7 Comparison of the proteolytic cleavage of PARP by cellular extracts from AHPN-treated Molt-4-neo and Molt-4-Bcl-2 cells. Cell lysates prepared from cells treated with and without AHPN (1 μ M) were analyzed for their ability to cleave 35 S-labeled tPARP (45 kD). The samples were subsequently analyzed by polyacrylamide gel electrophoresis, and fluorography performed. Cleavage products of 29 and 20 kD are indicated by arrows

damage or the Bcl-2 homologue Bak in rat fibroblasts (McCarthy *et al*, 1997) and Bax-induced cell death in Jurkat cells (Xiang *et al*, 1996). Z-VAD-FMK did not have any effect on the extent of the growth-inhibitory action of AHPN (Figure 8A). Flow cytometric analysis showed that treatment of Molt-4 cells with AHPN caused an accumulation of cells in the S-phase of the cell cycle. The presence of Z-VAD-FMK did not prevent this accumulation in the S-phase (not shown). These results suggest that growth arrest occurs independent of caspase activation and indicate that activation of caspase-3-like activity is an important factor in the execution of AHPN-induced cell death and appears to act in parallel with other events that by themselves can result in cell death.

Inhibition of apoptosis by Bcl-2

Members of the Bcl-2 family are major regulators of apoptosis (Korsmeyer, 1995; Kroemer, 1997; Reed, 1997). The induction of apoptosis by ceramide, Fas ligand, T cell antigen and dexamethasone in lymphocytes is inhibited by Bcl-2 (Hockenbery *et al*, 1990; Miyashita and Reed, 1992; Caron-Leslie *et al*, 1994; Zhang *et al*, 1996). We examined whether Bcl-2 expression could also inhibit AHPN-induced apoptosis. Using Molt-4 cells stably transfected with Bcl-2 or the control vector, AHPN induced death in Molt-4-neo cells as measured by Trypan Blue exclusion while cell death was greatly reduced but not completely blocked in AHPN-treated Molt4-Bcl-2 cells (Figure 9A). The inhibition of AHPN-induced cell death by Bcl-

2 could not be overcome by increasing AHPN concentrations (Figure 9B). Although Bcl-2 inhibited cell death, it did not block the growth-inhibitory effect of AHPN since treatment of Molt-4-Bcl-2 with AHPN still caused accumulation of cells in the early S-phase of the cell cycle as observed for AHPN-treated Molt-4 cells (not shown). Treatment of Molt-4 cells with AHPN did not alter the level of Bcl-2 mRNA or protein (not shown).

We next examined the effect of Bcl-2 on the AHPN-induced caspase-3-like activity in Molt-4-neo and Molt-4-Bcl-2 cells (Figure 10A). Bcl-2 greatly inhibited the activation of caspase-3-like activity by AHPN. In agree-

ment with these results, extracts from AHPN-treated Molt-4-Bcl-2 cells were unable to cleave tPARP (Figure 7B). The inhibitory effect of Bcl-2 was not limited to Molt-4 cells since expression of Bcl-2 also prevented the increase in caspase-3-like activity by AHPN in S49-Bcl-2 cells (Figure 10B). Inhibition of apoptosis was also evident from analysis of DNA fragmentation, as Bcl-2 inhibited the induction of internucleosomal degradation in S49-Bcl-2 cells by AHPN (Figure 2).

Overexpression of Bcl-2 also greatly inhibited the increase in annexin V-FITC binding induced by AHPN in Molt-4 cells (Figure 11). Our results demonstrate that overexpression of Bcl-2 inhibits several molecular events associated with the AHPN-induced cell death pathway.

Discussion

In this study, we demonstrated that treatment of several T cell lymphoblastoma cell lines with the novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid

Figure 8 Effect of Z-VAD-FMK on the inhibition of cell proliferation, induction of caspase-3-like activity, cell viability and DNA fragmentation. (A). Molt-4 cells were treated with DMSO or 1 μ M AHPN in the presence of Z-VAD-FMK at the concentrations indicated. After 24 h incubation total cell number, number of viable cells (Trypan blue-negative cells) and caspase-3-like activity were determined. The reduction in cell number was calculated as percent of the total cell number of DMSO-treated cells (□). The number of viable cells for each condition was calculated as percent of total cell number (▲). Protease activity was plotted as the percent of the activity present in AHPN-treated cells (no Z-VAD-FMK) (●). (B). Molt-4 cells were treated for 24 h with DMSO (NA) or 1 μ M AHPN in the presence or absence of Z-VAD-FMK. Cells were collected and analyzed by TUNEL assay

Figure 9 Bcl-2 inhibits the induction of cell death by AHPN in Molt-4 cells. A. Molt-4-neo and Molt-4-Bcl-2 were treated with AHPN (1 μ M) or DMSO (0.1%) and at different time intervals the number of alive and dead cells determined by Trypan blue exclusion. The percent alive cells was calculated and plotted. DMSO- (○) and AHPN-treated Molt-4-neo cells (●); DMSO- (□) and AHPN-treated Molt-4-Bcl-2 cells (■). (B). Cells were treated with AHPN at the concentrations indicated and after 24 h cell survival was determined. ●, Molt-4-neo cells; ■, Molt-4-Bcl-2 cells

Figure 10 Bcl-2 inhibits the activation of caspase-3-like activity by AHPN in Molt-4 and S49 cells. Cells were treated with AHPN (1 μ M) and at the indicated time points cells were harvested and DEVD-AFC cleavage activity measured in the corresponding lysates. (A). Molt-4 cells (hatched bars) and Molt-4-Bcl-2 (solid bars). (B). S49-neo cells (hatched bars) and S49-Bcl-2 cells (solid bars)

Figure 11 Bcl-2 inhibits the increase in annexin V binding by AHPN in Molt-4 cells. Molt-4-neo and Molt-4-Bcl-2 were treated with AHPN (1 μ M) or DMSO and after 17 h cells were harvested and analyzed for annexin V-FITC binding by flow cytometric analysis. Left panels, DMSO-treated cells; right panels, AHPN-treated cells. Top panels, Molt-4-neo; bottom panels, Molt-4-Bcl-2. Vertical axis indicates propidium iodide (PI) staining

(AHPN) caused growth arrest in the early S-phase of the cell cycle and induced apoptosis. The induction of apoptosis by AHPN was corroborated by the observed internucleosomal fragmentation of genomic DNA by 'ladder' or TUNEL assays, the increase in annexin V binding and the induction of caspase activity. Expression of Bcl-2 or the presence of the caspase inhibitor Z-VAD-FMK greatly reduced the rate of the execution of the apoptotic pathway but did not prevent the AHPN-induced cell cycle arrest or cell death. Our results suggest that AHPN triggers several parallel pathways that are involved in cell death.

AHPN has been shown to induce growth arrest and/or apoptosis in a number of carcinoma cell lines (Shao *et al*, 1995; Schadendorf *et al*, 1996; Oridate *et al*, 1997; Sun *et al*, 1997; Piedrafita and Pfahl, 1997; Hsu *et al*, 1997; Adachi *et al*, 1998). All-*trans* RA and a number of other retinoids that bind selectively to either the nuclear retinoid receptors RARs or RXRs, or exhibit selective anti-AP1 activity, did not affect proliferation of the lymphoma cell lines tested nor induced apoptosis or caspase activity. Although RT-PCR demonstrated expression of RAR α , β and γ and RXR α and γ mRNAs in Jurkat cells (Adachi H and Jetten AM, unpublished observations), the specificity by which these retinoids induce apoptosis suggests that AHPN acts through a novel mechanism that appears not to involve either RARE- or RXRE-dependent transactivation nor the anti-AP1 activity of retinoids (Shao *et al*, 1995; Sun *et al*, 1997; Piedrafita and Pfahl, 1997; Hsu *et al*, 1997; Adachi *et al*, 1998). This mechanism appears to be different from AHPN-induced apoptosis in thymocytes which is inhibited by RAR α -selective retinoids and appears to be mediated by RAR γ (Szondy *et al*, 1997). The mechanism of action of AHPN appears also different from that of 14-hydroxy-4,14-retroretinol which inhibits apoptosis in lymphocytes (O'Connell *et al*, 1996). It is possible that certain regulatory activities of retinoids are related to distinct conformational changes in the receptor as has been reported for the interactions of RA, 9-

cis-RA and the RAR α -selective antagonist RO 41-5253 with RAR α (Keidel *et al*, 1997). The induction of apoptosis by AHPN could be related to a distinct conformational change in the RAR γ receptor that is unique for this retinoid. However, several observations appear not to support a requirement for RAR γ in this action of AHPN. AHPN is a very effective inducer of apoptosis in promyelocytic leukemia HL-60 cells which do not express RAR γ (Hsu *et al*, 1997). In addition, the RAR-selective retinoid TTAB which was unable to induce apoptosis, binds to RAR γ with much greater affinity than AHPN and should be able to compete with AHPN for binding to this receptor and antagonize the induction of caspase activity by AHPN. However, no effect of TTAB on AHPN action was observed (Figure 6). It is interesting to note that the expression of several mRNAs regulated by AHPN identified thus far appears to be controlled at a posttranscriptional level (Shao *et al*, 1995; Adachi *et al*, 1998; Sakae *et al*, unpublished observations). These observations suggest that regulation at the level of mRNA stability may play an important role in the AHPN signaling pathway leading to apoptosis. Future studies have to provide further insight into these mechanisms.

Activation of caspases has been demonstrated to play an important role in the induction of apoptosis in mammalian cells by a variety of signals (Nicholson, 1994; Fernandes-Alnemri *et al*, 1994; Kumar and Flavin, 1996). Our results show that in the T cell lymphoma cell lines tested, activation of caspase-3-like and to a smaller degree caspase-1-like activity is part of the AHPN-induced cell death pathway in agreement with other studies (Hsu *et al*, 1997; Piedrafita and Pfahl, 1997). In both Jurkat and Molt-4 cells the activation of caspase(s) occurred after a lag of several hours. To study the role of this caspase activity in the execution of AHPN-induced apoptosis, the effect of the cell-permeable caspase inhibitor Z-VAD-FMK was examined. Treatment of Molt-4 cells with Z-VAD-FMK very effectively blocked the induction of caspase activity and DNA fragmentation by AHPN, and although the presence of Z-VAD-FMK slowed down cell death, it did not prevent it. In addition, the presence of Z-VAD-FMK had little effect on the inhibition of cell growth by AHPN or the accumulation of cells in the S-phase of the cell cycle. These results indicate that activation of caspase-3 accelerates the execution of AHPN-induced apoptosis and that other parallel events are sufficient to cause cell death.

A recent study (Thornberry *et al*, 1997) has defined the optimal specificities of a series of caspases. Z-VAD-FMK at high concentrations can inhibit many caspases, including caspase-1-like and caspase-3-like activity (Stefanis *et al*, 1996; McCarthy *et al*, 1997; Longthorne and Williams, 1997). Z-VAD-FMK has been reported to be able to inhibit several aspects of the apoptotic pathway; however, the effects on apoptosis are dependent on the type of signal and cell line tested. Both DNA fragmentation and phosphatidylserine externalization has been reported to require caspase activation and are inhibited by Z-VAD-FMK (Martin *et al*, 1996; Armstrong *et al*, 1996; Dubrez *et al*, 1996; Figure 8). Caspase inhibitors have been demonstrated to prevent Fas-induced apoptosis in Jurkat

cells; these cells also retain their proliferative ability (Longthorne and Williams, 1997). In contrast, Z-VAD-FMK blocked caspase activity, the increase in annexin V binding and DNA fragmentation in AHPN-treated lymphoma cells but did not prevent their accumulation in the S-phase of the cell cycle nor their cell death. A dissociation between caspase activation and growth arrest or apoptosis has been observed in several other cell systems (Slee *et al*, 1996; Stefanis *et al*, 1996; McCarthy *et al*, 1997; Monney *et al*, 1997). In PC12 cells, the inhibition of caspase activity by Z-VAD-FMK could be dissociated from its ability to inhibit cell death (Stefanis *et al*, 1996). In addition, inhibition of caspase activity by Z-VAD-FMK did not prevent cell death induced by oncogenes, DNA damage or the Bcl-2 homologue Bak in rat fibroblasts (McCarthy *et al*, 1997) or Bax-induced cell death in Jurkat cells (Xiang *et al*, 1996). These studies showed that Z-VAD-FMK prevented chromatin condensation, internucleosomal fragmentation, cleavage of PARP and externalization of phosphatidylserine. However, under these conditions surface blebbing and dramatic vacuolation still occurred. Moreover, in Bax-induced apoptosis Z-VAD-FMK did not prevent the reduction in mitochondrial potential. Whether such events still take place in cells treated simultaneously with AHPN and Z-VAD-FMK is being investigated. These observations suggest that although activation of caspases play an important role in carrying out at least part of the apoptotic process, cell death can occur independently from the activation of caspases inhibited by Z-VAD-FMK. Although the involvement of (an)other protease(s) cannot be ruled out, the specificity of such a putative protease should be different from that of caspase-1- and -3-like caspases. Recently, cathepsin D has been implicated in apoptosis suggesting a role for other proteases in this process (Deiss *et al*, 1996).

Bcl-2 has been demonstrated to play an important role in the regulation of apoptosis and to inhibit the induction of apoptosis in lymphocytes by a variety of signals (Hockenbery *et al*, 1990; Miyashita and Reed, 1992). Recent studies have shown that it plays a critical role in controlling the activation of caspases by regulating the release of cytochrome c from mitochondria indicating that Bcl-2 functions upstream of the caspase activation step in the cell death pathway (Kluck *et al*, 1997; Yang *et al*, 1997). In this study, we show for the first time that expression of Bcl-2 can inhibit the induction of caspase-3-like activity and apoptosis by AHPN. However, Bcl-2 did not reverse the inhibition of cell proliferation by AHPN and had little effect on accumulation of cells in the S-phase of the cell cycle. These results indicate that growth arrest can be uncoupled from apoptosis and that AHPN-induced growth arrest can occur independent of Bcl-2 action. AHPN had little effect on the hyperphosphorylation of Rb or the level of cyclin A in Molt-4 cells consistent with growth arrest in S-phase (Adachi H and Jetten AM, unpublished observations). These results contrast the reduction in Rb hyperphosphorylation and cyclin A levels associated with the G1-growth arrest observed in AHPN-treated lung and mammary carcinoma cells (Shao *et al*, 1995; Adachi *et al*, 1998). Why AHPN causes in different cell types growth arrest at different stages of the cell cycle has yet to be elucidated.

In summary, we demonstrated that AHPN is an effective inducer of apoptosis in a variety of T cell lymphoma cell lines. Induction of caspase-3-like activity plays an important role in the execution of AHPN-induced apoptosis but cells can undergo cell death in the absence of this activity suggesting that AHPN-induced cell death involves caspase-dependent and independent mechanisms. This action of AHPN appears to occur through a novel mechanism not involving RAR receptors and is therefore different from the RAR γ -dependent mechanism observed in thymocytes (Szondy *et al*, 1997). Future studies have to determine whether AHPN mediates its action through another receptor or binding protein. A recent study has shown that several derivatives of AHPN can inhibit growth of lung carcinomas in mice (Lu *et al*, 1997) indicating that this retinoid may be useful in cancer therapy and possibly in the treatment of lymphoblastoma as well. Elucidation of the AHPN-signaling pathway may lead to the development of new analogs with higher efficacy.

Materials and Methods

Materials

The retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN/CD437) was described previously (Delescluse *et al*, 1991) and obtained from Dr. U. Reichert (CIRD Galderma, Valbonne, France). The RAR- (SRI-6751-84/TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid), RXR- (SR11217, 4-[1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-methylpropenyl]benzoic acid), and anti-AP1-selective (SR11302, (E)-3-methyl-9-(2,6,6-trimethylcyclohexenyl)-7-(4-methylphenyl)-2,4,6,8-nonatetraenoic acid) retinoids were provided by Dr. M. Dawson (Fanjul *et al*, 1994). All-*trans* retinoic acid was obtained from Hoffmann-La Roche, Nutley, NJ, USA. Retinoids were dissolved in DMSO. Control cells received DMSO only.

Cell Culture

Human T cell lymphoma Jurkat (clone E6-1) and rat T cell lymphoma S49-neo and S49-bcl2 (Miyashita and Reed, 1992) were obtained from Drs Jeff Webster and Carl Bortner, NIEHS. Human T cell lymphoma Molt-4 and Molt-4-bcl2 were described previously (Zhang *et al*, 1996). All cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 μ g streptomycin (complete media). The T-cell hybridoma cell line DO11.10 was provided by Dr. B.A. Osborne (University of Massachusetts, Amherst, MA, USA) and grown in RPMI-1640 medium supplemented with 10% horse serum.

MTT assay

Cells (1.0×10^6 cells/ml) were plated in 24 well dishes (0.5 ml/well) and treated with and without AHPN as indicated. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Boehringer Mannheim) was used to analyze cell proliferation and viability. MTT solution (100 μ l) was added to the cell suspensions at a final concentration of 0.5 mg/ml. After incubation for 1 h at 37 $^{\circ}$ C, 0.5 ml solubilization buffer was added and 24 h later the absorbance measured at 575 and 690 nm. The difference between these two values is a measurement for cell survival.

DNA fragmentation assay

Untreated cells and cells treated with 1 μ M AHPN for 8 h were collected by centrifugation. Cells were washed once in cold PBS, resuspended in 100 μ l lysis buffer A (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% sodium laurel sarcosine and 1.8 mg/ml proteinase K (Boehringer Mannheim) and incubated overnight at 50°C. Then 50 μ l of RNase (RPA grade; 1 mg/ml, Ambion, Austin, Texas, USA) was added and the mixture incubated for an additional two hours at 37°C. After the addition of 10 $\times$ loading buffer (50% glycerol, 0.006% bromophenol blue, 0.006% xylene cyanol FF), the DNA sample was electrophoresed in 1% agarose gel with 1 $\times$ TBE buffer as described (Zhang *et al*, 1995). DNA ladders were visualized by ethidium bromide staining.

Flow cytometric analysis

TUNEL assay. Jurkat cells (1×10^5 /100-mm dish) were plated in complete RPMI1640 medium and 2 days later treated with 1.0 μ M AHPN for 8 h. Flow cytometric analysis of DNA fragmentation was performed using a variation of the nick end-labeling technique of Gavrieli as described (Gavrieli *et al*, 1992), substituting dUTP-fluorescein isothiocyanate (FITC) for biotinylated dUTP in the labeling reaction. Briefly, the cell pellets were washed with cold PBS and fixed with 1% paraformaldehyde in PBS for 15 min. The cell pellet was washed in PBS, permeabilized using 70% ethanol, washed again in PBS containing 1% bovine serum albumin, and labeled. Cell samples were divided into two halves. One half was used to determine background fluorescence by omitting the terminal deoxynucleotidyl-transferase enzyme (TdT) from the reaction mixture. TdT was added to the second half of the sample and increases in fluorescence (FL1) were evaluated by flow cytometric analysis. Analyses were carried out with the Flow Cytometric Analysis Reagent Set (Boehringer Mannheim). Clumps and doublets were excluded from the analysis by gating on forward scatter versus propidium iodide (PI) fluorescence (FL2-width). Flow cytometric analysis was performed with a Becton Dickinson FACSsort and accompanying CellQuest software.

BrdU analysis. For the analysis of BrdU incorporation and PI labeling, cells were pulse-labeled for 30 min with 10 mM BrdU, washed in ice-cold PBS and pelleted. Cell pellets were resuspended in PBS and cells fixed in ice-cold ethanol as described previously (Adachi *et al*, 1998). Incorporation of BrdU was measured using an FITC-conjugated anti-BrdU antibody (Becton Dickinson) and the Flow Cytometric Reagent Set.

Annexin V binding. AHPN-treated and untreated cells were collected by centrifugation and washed once with cold PBS. The analysis of annexin V binding was carried out using the TACSTM Annexin V-FITC kit from TREVIGEN (Gaithersburg MD, USA) according to the manufacturer's instructions. Cells were resuspended, divided equally over four eppendorf tubes and then centrifuged. After removing the supernatant, each cell pellet was gently resuspended in 100 μ l of binding reagent with or without propidium iodide and/or annexin V-FITC and incubated at room temperature in the dark. After 15 min, 400 μ l of 1X binding buffer was added and samples analyzed by flow cytometry.

Caspase fluorogenic enzyme assay

Cells treated with AHPN and untreated cells (3×10^7 cells each) were harvested by centrifugation and then lysed on ice for 3 min in 100 μ l

10 mM MgCl₂, 0.25% NP-40 (lysis buffer B). Cell debris was removed by centrifugation at 213 000 $\times g$ in a TL-100 centrifuge for 30 min at 4°C. The caspase-1-like and caspase-3-like activity in the supernatants were measured by a fluorometric assay (). All peptides were purchased from Biomol, Plymouth Meeting, PA, USA. Enzyme assays contained 30 μ l of supernatant and 50 μ M Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) or Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin (YVAD-AFC) substrate in buffer (50 mM HEPES, 10% sucrose, 0.1% CHAPS, 100 mM DTT, pH 7.5) with or without 12.5 μ M of the reversible inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) or Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO), respectively. Samples were incubated at 30°C, and after 5 and 65 min their fluorescence were measured at 505 nm (excitation 400 nm) using a Perkin Elmer 650-40 Fluorescence Spectrophotometer. A standard curve of fluorescence vs free AFC (Sigma Chemical Co., St. Louis, MO, USA) was used to calculate the specific caspase activity. The cell-permeable, irreversible caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD-FMK) was used to study the effects of caspase inhibition on the cell death pathway in cultured cells. Cells were washed extensively to remove free Z-VAD-FMK before cellular extracts were prepared.

Poly(ADP-ribose)polymerase (PARP)-cleavage assay

A PET15 vector containing a cDNA encoding the first 338 amino acid N-terminal region of PARP including the caspase-3 cleavage site, was obtained from Dr J Collier (Harvard University). The TNT-coupled reticulocyte lysate system (Promega) in combination with T7 polymerase and ³⁵S-methionine (Dupont NEN) was used to synthesize radiolabeled 48 kD truncated PARP (tPARP). The radiolabeled products were incubated with cellular extracts (5 μ g protein) in buffer C (100 mM Hepes pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT) at 37°C for different time periods. The reaction was stopped by the addition of sample buffer. Samples were subsequently analyzed by SDS-polyacrylamide electrophoresis. The gel was dried and autoradiography performed.

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