Retro-Retinoids in Regulated Cell Growth and Death

By Mary Jane O'Connell,* Ramon Chua,* Beatrice Hoyos,* Jochen Buck,‡ Yanqiu Chen,‡ Fadila Derguini,* and Ulrich Hämmerling*

*From the *Program in Immunology, Memorial Sloan-Kettering Cancer Center, and the ‡Department of Pharmacology, Cornell University Medical College, New York 10021*

Summary

Vitamin A serves as a prohormone from which three classes of active metabolites are derived: the aldehydes, the carboxylic acids, and the *retro*-retinoids. Although these three classes are united under the rubric of signal transduction, they act by different molecular mechanisms: the 11-*cis*-retinaldehydes combine with opsin to form the universal visual pigments and the retinoic acids form ligands for transcription factors, whereas the *retro*-retinoids, as shown here, intersect with signal transduction at a cytoplasmic or membrane site. The *retro*-retinoid, anhydroretinol (AR), has long been known to act as a growth inhibitor in lymphocytes, whereas 14-hydroxy-4,14-*retro*-retinol (14-HRR) is required for normal lymphocyte proliferation. A mutually reversible relationship exists between these two *retro*-retinoids as one can reverse the effects of the other when given in pharmacological doses. The common explanation for reversible inhibition is competition for a shared receptor. We now provide evidence that when AR is given to T cells unmitigated by 14-HRR, rapid cell death can occur. The circumstances are closely related to nonclassical forms of apoptosis: within 2 h of AR administration the T cells undergo widespread morphological changes, notably surface blebbing and ballooning and, inevitably, bursting. In contrast, nuclear changes are comparatively mild, as indicated by absence of chromatin condensation and overt DNA cleavage to discrete nucleosomal fragments, although DNA nicks are readily discernible by terminal deoxynucleotidyl transferase assay. What further distinguishes the AR-induced form of apoptosis from classical ones is a lack of requirements of messenger RNA and protein synthesis, suggesting that the events leading to cell death are primarily initiated and play themselves out in the cytoplasm. This view is further reinforced by the finding that herbimycin A can prevent the onset of programmed cell death. The importance of our findings is that they strongly suggest a second messenger role for vitamin A metabolites in the cytoplasmic realm that has not been seen previously. These findings are entirely compatible with a general notion that in a cell requiring multiple coordinated signals for survival, the provision of an unbalanced signal can initiate programmed cell death. Collectively, our data also challenge the paradigm that retinoids (outside vision) solely mediate their function via the steroid/retinoic acid receptor family of nuclear transcription factors. Instead, a mode of action in the cytoplasmic realm akin to one attributed to other small lipophilic second messenger molecules, such as diacyl glycerol or ceramide, may apply to *retro*-retinoids.

When resting lymphocytes are stimulated through their antigen receptors, they initiate a complex system of signal transmission and amplification that employs several parallel, though coordinated, pathways. The end result is a reprogramming of the nucleus with consequent structural and functional changes, initiation of proliferation being one of them. Transformed lymphocytes proliferate constitutively, but are as dependent on functioning signal pathways as their normal counterparts. They grow because their signal apparatus linked to cell cycle control is permanently turned on owing to mutations in one or more components (1, 2). Most signaling pathways are unaffected by these mutations.

Intracellular signal events are not only mediated by phosphokinases and phosphatases but also intersect with enzymes that generate chemical mediators. Examples include small lipophilic molecules, such as diacyl glycerol (3) and ceramide (4). These molecules are believed to activate mediator-specific phosphokinases and feed back into signal cascades whose upper portion may have triggered their synthesis. Thus, these lipophilic second messenger molecules may serve as amplification devices.

The metabolites of vitamin A (retinol, Fig. 1, *structure 1*) belong to the family of lipophilic mediators. The widely studied metabolite, retinoic acid (Fig. 1, *structure 2*), has been linked to differentiation in different cellular systems

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(5–7). The differentiated phenotype is triggered upon binding of retinoic acid to specific nuclear receptors on the promoters of pertinent genes (8, 9). Retinoic acid action is thus related to that of steroids and thyroid hormone. However, control of differentiation is only one of the functions carried out by vitamin A, in addition to its role in vision. Retinol is also involved in controlling proliferation in several cellular systems (10–13). Interestingly, retinoic acid does not substitute for retinol in the cell growth assay. Instead, other metabolites appear to be used in the role of regulator of cell growth, as described for mitogen-activated normal and transformed lymphocytes (12). The growth regulatory function has been traced to 14-hydroxy-4,14-*retro*-retinol (14-HRR[1], Fig. 1, *structure 3*) (11, 14).

That 14-HRR, like retinoic acid, appears to engage a cellular receptor followed from pharmacological studies with the naturally occurring structural analog, anhydroretinol (AR, Fig. 1, *structure 4*). AR inhibits the growth-promoting activity of 14-HRR, and this negative effect can be reversed by increased doses of 14-HRR (15, 16). Eventually, however, given high enough doses, AR displaces 14-HRR completely and initiates a signal that leads to rapid cell death. Based on the paradigm of reversible inhibition, the best assumption is that 14-HRR and AR bind to the same receptor, neither the location nor the nature of which is presently known, although explanations involving multiple receptors are possible. By analogy with retinoic acid one might imagine a nuclear receptor, but on the basis of the findings presented below, this may not be so. We show that the cell death signal triggered by AR is not mediated by induced RNA transcription and protein synthesis, but involves a signaling step leading to catastrophic morphological changes and damage to DNA akin to nonclassical apoptosis. We conclude that the action of AR, unmitigated by its positively acting counterpart, 14-HRR, can induce programmed cell death. Furthermore, by inference the putative receptor for 14-HRR/AR may be cytoplasmic or cell membrane associated, and therefore the paradigm that retinoids outside vision mediate their function solely via ligand-assisted transcription may have to be amended.

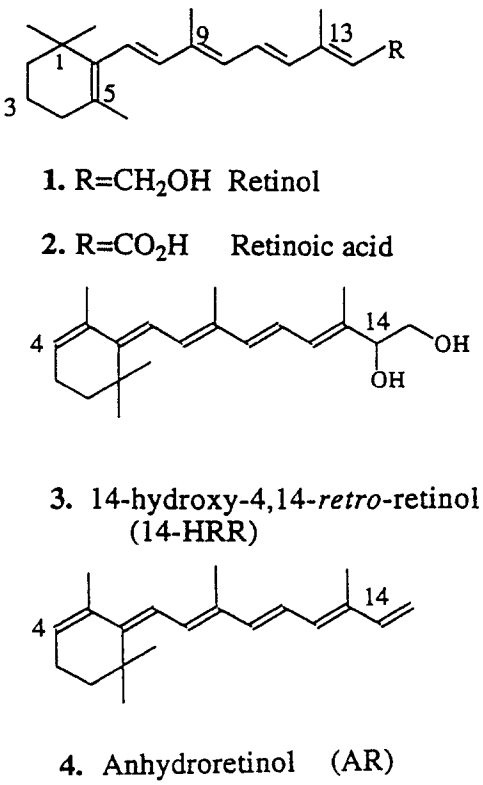

Figure 1. Structures.

Materials and Methods

Cell Lines. The murine T cell line, ERLD, was derived from a thymoma of C57Bl mice at Sloan-Kettering Institute (17). The Epstein-Barr virus–transformed human B lymphoblastoid cell 5/2 was established from the blood of a healthy volunteer. Both cell lines were serially passaged in RPMI-1640 medium supplemented with 7% fetal calf serum and antibiotics.

Reagents. Herbimycin A, methyl 2,5-dihydroxycinnamate, lavendustin A, staurosporin, genistein, actinomycin D, all-trans retinol, and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). and stored as concentrated stock solutions at −70°C.

Retinoids. 14-HRR and AR were synthesized as reported (14, 16). Both retinoids were purified by reversed phase HPLC and stored as methanolic solution under argon at −70°C.

Cell Culture Experiments. ERLD or 5/2 cells were transferred to serum-free insulin, transferrrin, linoleic acid, and bovine albumin (ITLB) medium (2) and plated in microtiter plates with the drugs and retinoids as specified in the figure legends. At the indicated time points, cell samples were counted in the presence of trypan blue under an inverted microscope. The close spacing of time points with multiple parallel cultures precluded counting replicate cultures. However, each experiment was repeated at least three times and yielded the same basic data.

TdT Assay for DNA Damage. Measurement of DNA nicking was performed with minor modifications according to Gorczyca et al. (18), *viz.*, incorporation of biotinylated-dUTP (b-dUTP) at free 3′ OH ends of nicked DNA by TdT followed by FITC-avidin staining. Log-phase ERLD murine T leukemia cells were preincubated with actinomycin D, cycloheximide, or herbimycin A, and subsequently treated with varying concentrations of retinoids as indicated in the legend to Fig. 4 *B*. At specified time intervals, aliquots of cell samples were collected and fixed in 1% formaldehyde/PBS/0.1% Triton X–100 on ice, washed once with PBS, resuspended in cold 70% ethanol/PBS, and stored at 4°C. After rehydration, cells were resuspended in cacodylate buffer, 25 mM $CoCl_2$, and 0.5 nmol b-dUTP in the presence or absence of 10 U TdT enzyme (Boehringer Mannheim Biochemicals, Indianapolis, IN). After a PBS wash, cells were resuspended in FITC-avidin staining buffer containing 4× SSC, 2.5 μg/ml FITC-avi-

[1] *Abbreviations used in this paper:* AR, anhydroretinol; b-dUTP, biotinylated dUTP; ERLD, E-strain, radiation-induced leukemia D; 14-HRR, 14-hydroxy-4,14-*retro*-retinol; JTLB, insulin, transferrin, linoleic acid, and bovine albumin medium.

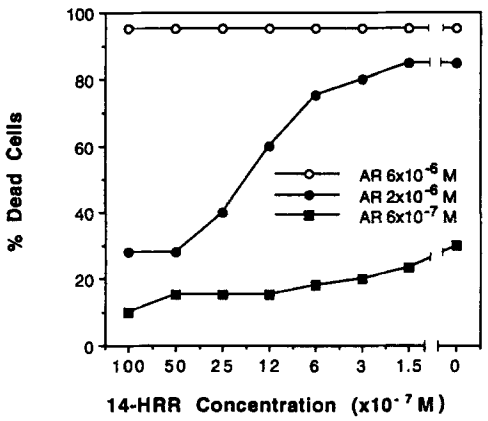

Figure 2. Inhibition of AR-induced cytotoxicity by 14-HRR. ERLD cells were simultaneously treated with different concentrations of AR and serially diluted 14-HRR. Ratios of live and dead cells were determined after 4 h of culture by trypan blue exclusion.

din (Boehringer Mannheim Biochemicals), 0.1% Triton X–100 (Sigma Chemical Co.) and 5% non-fat dry milk. After a 30-min incubation in the dark, cells were washed in PBS/0.1% Triton X–100 and resuspended in PBS containing 5 μg/ml propidium iodide, 0.1% RNase A (Sigma Chemical Co.) and 0.1% Triton X–100. DNA content vs. dUTP incorporation of individual cells (~20,000 gated events per sample) was measured by respective red and green fluorescence on a FACScan® flow cytometer using Celquest data analysis (both from Becton Dickinson & Co., San Jose, CA) and Multicycle statistical analysis software (Phoenix Flow Systems, San Diego, CA).

Results

We have previously reported that 14-HRR and AR display a mutually antagonistic relationship in regard to regulation of the growth of human lymphoblastoid cells and receptor-mediated activation of murine T cells (15). We showed that the entire dose–response curves of 14-HRR could be shifted to higher concentration ranges by simultaneously increasing the concentration of added AR. We interpreted these results of reversible inhibition as evidence for a common receptor for which these two retinoids compete. The T cell line, ERLD, and the lymphoblastoid B cell line, 5/2, are exquisitely sensitive to AR. At a concentration range of 6×10^{6} M to 6×10^{-7} M, this drug led to rapid cell death (Fig. 2). AR acted presumably by overriding the endogenous retinol metabolite, 14-HRR. Cytotoxicity was reversible by 14-HRR (shown with ERLD in Fig. 2) or its precursor, retinol (data not shown). Retinoic acid was inert in this assay system, neither inducing nor preventing cell death (data not shown). Therefore, the reversible inhibition observed previously in the long-term tissue culture experiments with normal T, lymphoblastoid 5/2, and promyelocytic HL-60 cells (13, 15) also holds true in the newly developed short-term culture system.

AR-induced cell death was not distinguishable from the cell cycle–independent cell death we previously described consequent to retinol deficiency (19). In either case, the cells showed a disintegrating cytoplasm with fused vacuoles whereas the nuclei, with their nucleoli, remained intact for prolonged periods of time. AR administration appeared to accelerate this process in strict dose dependency, inducing at the maximum dose used widespread surface blebbing, ballooning, and osmotic bursting of cells within 2–4 h (Fig. 3). However, neither the degradation of DNA to oligonucleosomal bands (Fig. 4 *A*) nor the chromatin condensation typical for apoptosis (not shown) was evident. Nevertheless, as a result of exposure to AR, the DNA was damaged, displaying multiple nicks that were detected by the sensitive TdT reaction (Fig. 4 *B*). Importantly, however, microscopic inspection in the presence of the vital dye, trypan blue, revealed that the widespread cellular swelling and osmotic damage occurred simultaneous to or even before DNA damage.

That 14-HRR and retinol rescued cells from AR-induced cytotoxicity (Fig. 2) argued against trivial membrane toxicity. Furthermore, the rapid onset of cellular damage made a nuclear mechanism unlikely, as the 1–2-h time span from the administration of AR to the appearance of cytosolic cell swelling would be relatively short for a sig-

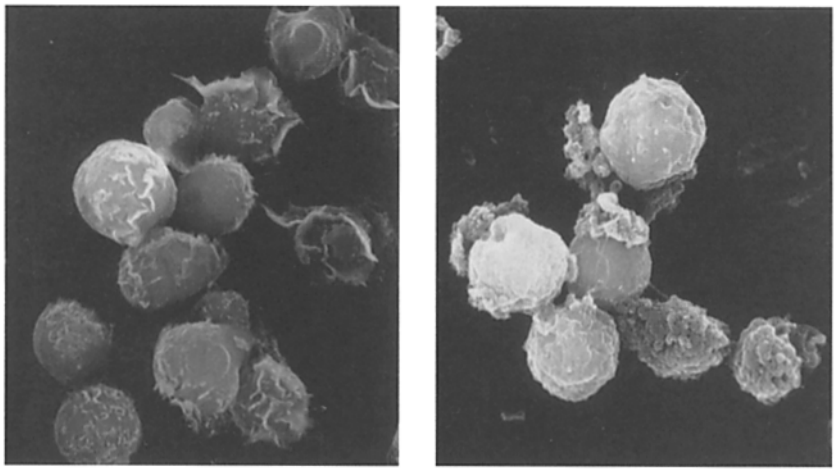

Figure 3. Morphological changes associated with AR-induced cytotoxicity. ERLD cells were cultured in the presence of 2×10^{-6} M anhydroretinol. At the onset of culture, scanning electron micrographs revealed the normal appearance with numerous surface ruffles (*left*). 3 h later, most cells had lost their ruffled appearance, exhibited numerous blebs, looked bloated, in part took on a cratered appearance, and began to leak amorphous material (*right*). By trypan blue uptake, most cells were no longer osmotically intact.

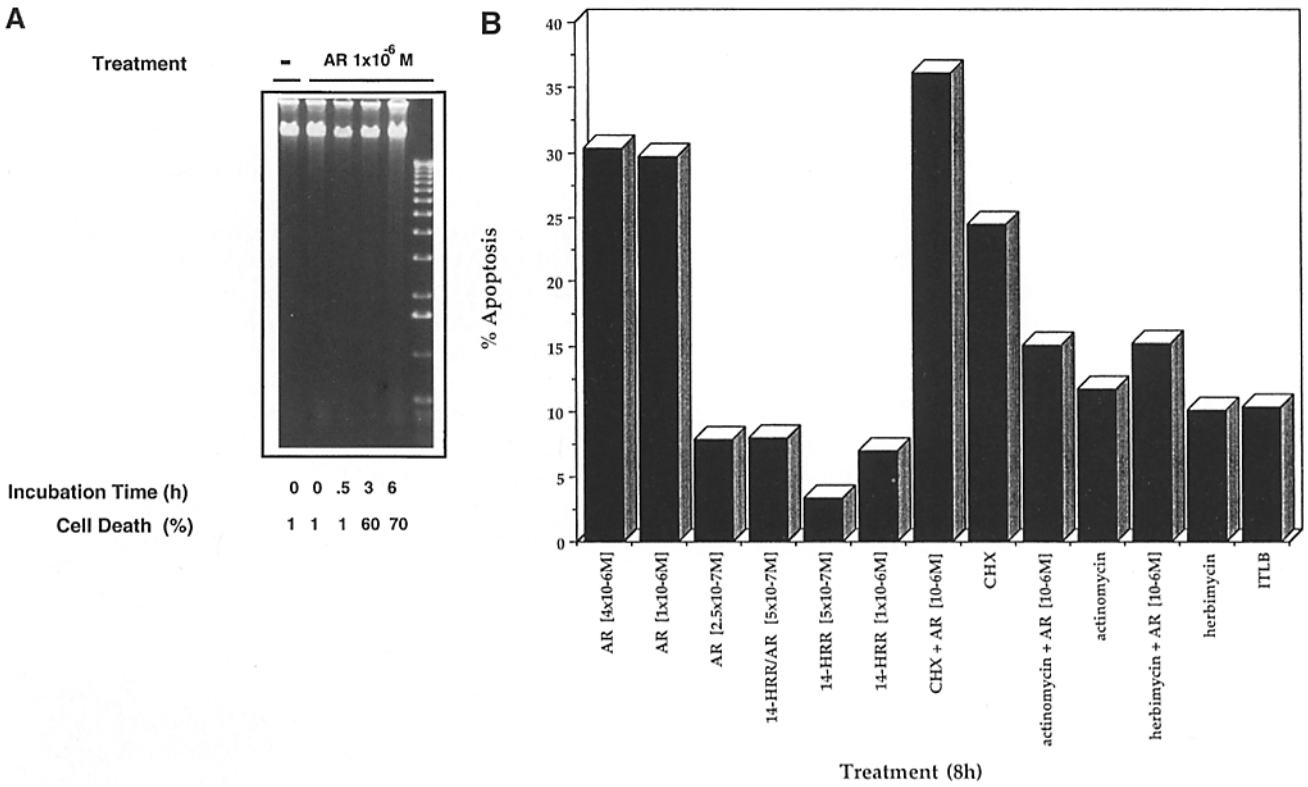

Figure 4. (*A*) Absence of DNA fragmentation in AR-induced apoptosis. ERLD cells were cultured in the absence (lane *1*) or presence (lanes *2–5*) of 10^{-6} M AR. Regardless of treatment modality of time, the integrity of DNA was completely preserved, although at the 3- and 6-h time points (lanes *4* and *5*), most cells were dead by trypan blue exclusion. (*B*) TdT assay for DNA damage. In rapidly proliferating ERLD murine T leukemia cells, induction of apoptosis in cumulative effect is measured by TdT incorporation of biotin-labeled dUTP/FITC-avidin at 3′ OH termini of nicked DNA. Cell samples were preincubated with actinomycin D (3 μg/ml), cycloheximide (10 μg/ml), herbimycin A (2 μg/ml), or ITLB medium alone. Preincubated and additional test samples were then supplemented with retinoids, AR or 14-HRR, at the concentrations shown. Aliquots were collected at time intervals of 2, 4, 8 (shown here), or 24 h. Cells were fixed, stained, and assayed for DNA content vs. dUTP incorporation by red/green fluorescence measurement by flow cytometry as described in Materials and Methods.

nal to be initiated in the nucleus, exported to the cytoplasm, and implemented there. Retinoids, on the other hand, have been squarely tied to a nuclear mechanism of action, obeying the paradigm of ligand-assisted transcriptional activation (20, 21). Whereas *retro*-retinoids have been tested for their ability to act as ligands for RARs, RXRs, and orphan receptors, none has so far shown any transactivation of direct binding activity (Buck, J., R. Evans, U. Hämmerling, D. Mangelsdorf; F. Derguini, and R. Heyman, unpublished results). To clarify whether or not new RNA transcription and protein synthesis were required for the induction of cytotoxicity, ERLD cells were pretreated with actinomycin D or cycloheximide before AR was applied. Neither drug showed any sign of inhibition of AR-induced cytotoxicity (Fig. 5, *A* and *B*), although actinomycin D inhibited the AR-induced DNA damage observed in the TdT assay (Fig. 4 *B*). Indeed, not surprisingly, cycloheximide at doses >1 μg/ml slightly accelerated cell damage inflicted by AR, as well as causing moderate apoptotic damage on its own (Fig. 4 *B*). Evidently then, macromolecular synthesis was of secondary importance, and the main events appeared to be confined to the cytoplasm. These considerations brought up the question of how a lethal signal could be delivered, if indeed signaling was involved at all. Since our previous work had shown 14-HRR to be a contributing factor in T cell activation (12), we decided to test different pharmacological blockers of signal transduction. Staurosporin, an inhibitor of protein kinase C (22), and the tyrosine kinase inhibitors, methyl 2,5-dihydroxycinnamate, lavendustin A, and genistein (23), were inert in the assay system used (data not shown). However, herbimycin A, a putative inhibitor of *src*-like phosphotyrosine kinases, prevented AR-induced cell death (Fig. 6). During the observation period of 8 h, herbimycin A at 3 and 1 μg/ml was able to efficiently, and at 0.3 μg/ml to partially, suppress the AR effect.

Discussion

Of the three classes of retinol metabolites, i.e., the aldehydes, the carboxylic acids (all *trans* and 9-*cis* retinoic acids), and the *retro*-retinoids (11), the mechanisms of action have been delineated with certainty for the first two. In the first class, 11-*cis*-retinaldehyde combines with opsin to form the universal visual pigment. Excitation by light triggers the phototransduction cascade that ultimately leads to de-

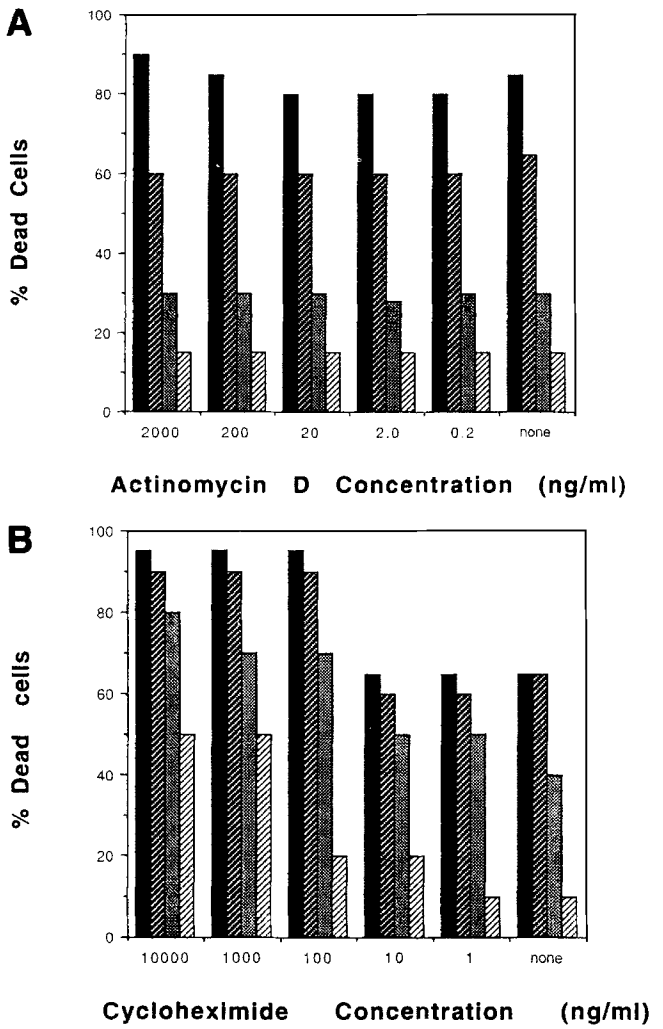

Figure 5. Failure of actinomycin D (*A*) and cycloheximide (*B*) to inhibit AR-induced cell death. ERLD cells were incubated for 30 min with indicated doses of actinomycin D (*A*) or cycloheximide (*B*) and then cultured with the different concentrations of AR. 4 h later, differential cell counts were performed in trypan blue. ■, AR 6×10^{-6} M; ▨, AR 2×10^{-6} M; ■, AR 6×10^{-7} M; ▨, none.

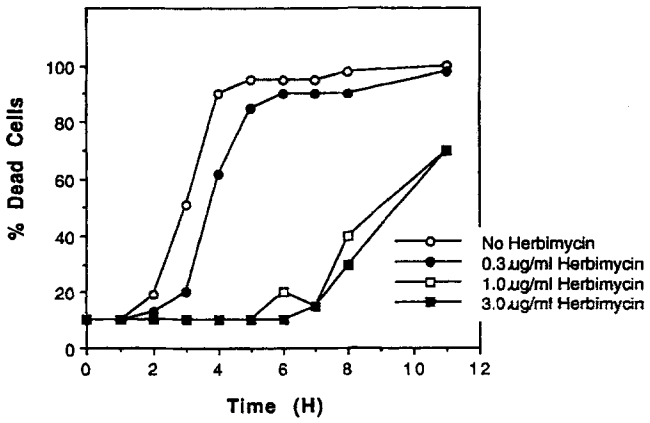

Figure 6. Inhibition of AR-induced cell death by herbimycin A. ERLD cells were cultured with 2×10^{-6} M AR and the indicated concentration of herbimycin A. Dead/live ratios were followed over time by differential counting in trypan blue.

creased channel conductivity and a nerve impulse. This is an example, par excellence, of inside-out signal transduction. In the second class, retinoic acids mediate signals from the extracellular environment to the transcripitional apparatus by direct passage into the nucleus and binding to the preexisting nuclear receptors. In the third class, *retro*-retinoids, 14-HRR, and AR are also implicated in signal transduction, although their receptors are not known. Operationally, they belong to the group of small lipophilic second messenger molecules, since they are synthesized in the same cell that they purportedly regulate (irrespective of the fact that because they can easily traverse biomembranes they might also operate in paracrine fashion). 14-HRR is widely distributed in the body, and it appears that virtually all dividing cells synthesize this metabolite. The analysis of a panel of tissue culture cell lines representative of a wide variety of tissues has revealed none that was unable to metabolize tritiated retinol to 14-HRR (O'Connell, M.J., and U. Hämmerling, unpublished results). Moreover, 14-HRR is evolutionarily conserved from insects to humans. Functionally, it has been consistently linked to cell proliferation (12), as 14-HRR has proven to be of critical importance for activation of resting normal cells and for maintenance of cycling cells in a number of cases. The best evidence for this is the ability of 14-HRR at physiological concentrations to reverse the cell cycle arrest brought on by vitamin A withdrawal in several cell lines. Retinoic acid is inactive in that respect. How this growth regulation may be effected, what molecular targets may be involved, and especially what might be the nature of the primary receptor(s), are unclear. That there is a receptor can be inferred from pharmacological studies of an antagonist of 14-HRR, AR (15, 16). We have shown previously that the growth-promoting activity of 14-HRR can be completely and reversibly inhibited by AR, strongly suggesting competition of binding to the same molecule. Other explanations are conceivable in which two independent receptors are invoked, necessitating convergence of compensating signal pathways at some downstream point.

In our experiments we have used cell death or DNA damage induced by AR as biological readouts. ERLD is a cell line derived from a murine T cell lymphoma that is fortuitously of exceptional sensitivity to AR. This is by no means unusual as many cell lines of murine or human origin react to AR with a gradation of consequences, ranging from growth retardation to full arrest and frequently to cell death. As vitamin A starvation in these same cells produces similar symptoms of growth arrest and cell death, although by slower kinetics, it is possible that the critical issue is the availability of 14-HRR. If so, the action of exogenous AR may be to antagonize the endogenous 14-HRR, whereas in retinol starvation, 14-HRR synthesis may be gradually curtailed for lack of biochemical precursor. This concept has been tested previously in vitamin A–deprived HL-60 cells where cell viability was found to be sustained as long

as intracellular retinyl esters were available as substrate for 14-HRR biosynthesis and preservation of a constant 14-HRR level. The eventual exhaustion of this intracellular store of vitamin A coincided precisely with a decline of the intracellular 14-HRR concentration, loss of cell proliferation, and cell death (13).

The important question is whether AR, unmitigated by 14-HRR, activates programmed cell death. Several observations indicate that this may be the case. Foremost are morphological changes, including surface blebbing and widespread osmotic damage (Fig. 3). Second, the data in Fig. 4 indicate that nicks in the DNA abound after exposure to AR. These symptoms are commonly interpreted as signs of apoptosis. Yet we have not observed two other manifestations of classical apoptosis: there was neither chromatin condensation nor nucleosomal fragmentation of DNA (Fig. 4 *A*). Rather, the overall picture fits more the nonclassical apoptosis associated with perforin-induced cytotoxicity (24, 25).

The reaction of ERLD cells to AR is among the most fulminant ones seen to date, and the short time span of only 1–2 h, in which morphological changes such as surface blebbing, ballooning, and eventually cell rupture occur, alerted us to the possibility that the sequence of events was mostly cytoplasmic. Importantly, the morphological damage seemed to occur with the same kinetics as DNA damage, yet did not depend on RNA and protein synthesis (Fig. 5), implying a cycle of events triggered mainly in the cytoplasm and perhaps not stemming from nuclear damage. In fact, there is no compelling reason to think that nuclear damage spotted by the TUNEL technique is the cause of cell death, in particular since the fate of cells was sealed whether transcription or translation was active or not. The observed DNA damage could be ancillary to, or even a consequence of, a catastrophic event triggered in the cytoplasm by a pharmacological dose of AR.

Assuming that *retro*-retinoids act as second messengers, a precedent for the induction of apoptosis by small lipophilic messengers exists. It has been reported that ceramide, when given to cells as the sole stimulator, can induce cell death by apoptosis. The pertinent point is that ceramide is known to regulate certain phosphokinases (4), and by analogy, the 14-HRR/AR pair of agonists/antagonists may do so, too. The reversal of AR effects by herbimycin A is also consistent with the notion that AR is involved in signaling, acting perhaps as a second messenger. Herbimycin A can reverse the transformation by the oncogenes *src*, *fps*, and *abl* but is not effective against the transformation by *ras*, *raf*, and *myc* (26). It also inhibits signal transduction by the T cell antigen receptor (27). Contrary to lavendustin A and genistein, herbimycin A does not bind to the ATP-binding domain of tyrosine kinases but reportedly inhibits the association of $p60^{v\text{-}src}$ with the cytoskeletal structure and with phosphatidylinositol 3′-kinase and may exert its effect on SH2 functions (28). Recently, it was shown that herbimycin A can induce apoptosis in several cellular systems (29, 30) possibly by preventing the upregulation of bcl-2 mRNA. It is striking that one and the same drug can in one context prevent cell death induced by AR, as shown here, and in another induce apoptosis.

Although the regulatory circuits negatively affected by AR and resulting in lethality may not be identical with those functioning in growth regulation, they appear to be connected with the same receptor. This follows from the ability of 14-HRR to reversibly block the lethality induced by AR (Fig. 2), exactly as reported previously for growth regulation. Hence, we can assume that the cell death signal is initiated by AR at a similar site as the growth signal transmitted by 14-HRR, although the resulting cascades diverge and lead to totally different events. Besides demonstrating the involvement of vitamin A in the regulation of cell vitality, our results speak also to the issue of its mechanism of action. Although this is frequently equated with retinoic acid, the events described here are clearly separate from those mediated by retinoic acid. They involve different vitamin A metabolites, the *retro*-retinoids, and, inasmuch as they appear to be located in the cytoplasmic realm and not the nucleus, different receptor molecules. To recapitulate, two criteria support our conclusion that the AR/14-HRR receptors are cytoplasmic, *viz.*, AR-induced cell death is independent of macromolecular synthesis, i.e., cytotoxicity incurred by AR is insensitive to actinomycin D and cycloheximide, and DNA damage induced by AR is inhibitable by herbimycin A. This is an important conclusion as, to date, retinol action has only been seen in the nucleus, by activation of RAR or RXR receptors with retinoic acid, leading to increased transcription. The mechanism of action of *retro*-retinoids is therefore very likely different from the transcriptional regulation attributed to retinoic acid.

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Address correspondence to Dr. Ulrich Hämmerling, Immunology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

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