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INDUCTION OF APOPTOSIS BY ACYCLIC RETINOID IN THE HUMAN HEPATOMA-DERIVED CELL LINE, HUH-7

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HuH-7 cells, a human hepatoma-derived cell line, underwent apoptosis in response to all-*trans* 3, 7, 11, 15-tetramethyl- 2, 4, 6, 10, 14- hexadecapentaenoic acid, or acyclic retinoid. The retinoid-induced apoptosis was verified by a characteristic step-wise fragmentation of genomic DNA and chromatin condensation. The induction of apoptosis was detected as early as 8 hrs after the addition of the retinoid and was concentration dependent(0.1~10 μM). Neither the natural retinoid all-*trans* retinoic acid nor 9-*cis* retinoic acid induced apoptosis. These data strongly indicate that the antitumor activity of the acyclic retinoid may be partly explained by the induction of apoptosis in tumor cells.

Retinoids inhibit tumor development and induce the differentiation of malignant cells, in addition to playing key biologic roles in embryonal morphogenesis, epithelial cell differentiation,and the terminal differentiation of blood cells (1). Although the molecular mechanisms underlying the antitumor action of retinoids have yet to be clarified, the antagonistic effect of retinoids against tumor promoters such as phorbol esters is the most probable mechanism, at present, to explain how retinoids suppress tumorigenesis (2).

On the other hand, retinoids may well promote apoptosis and this may underlie their activity as anticarcinogenic agents. Recently, cytokine-induced apoptosis has been shown to be operative in some malignant tumor cells (3,4). For example, apoptotic cell death was induced in Hep 3B hepatoma cells by treating them with transforming growth factor-ß1 (TGF-ß1) (5). In addition to cytokines, analogs of membrane lipid

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metabolites, such as C_2-ceramides (3) and 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3), have been reported to trigger apoptosis in HL-60 cells (6). The latter phospholipid has been shown to be an anticarcinogenic agent.

We have defined all-*trans* 3, 7, 11, 15-tetramethyl- 2, 4, 6, 10, 14-hexadecapentaenoic acid as acyclic retinoid, one of the most potent ligands for cellular retinoic acid-binding protein (CRABP) (7). This acyclic retinoid has been shown to reduce the incidence of mouse skin carcinoma induced by 7, 12-dimethyl benz[a]anthracene and croton oil and also to prevent spontaneous hepatocarcinogenesis in C3H/HeNCrj mice (7). The lower toxicity of this retinoid, probably because it is metabolized as a polyunsaturated fatty acid, makes it an attractive chemoprevention agent for clinical trials.

In this paper, we examine whether acyclic retinoid might induce apoptotic cell death in hepatoma cell lines, by measuring the pattern of DNA fragmentation and chromatin condensation.

Materials and Methods

Materials. All-*trans* retinoic acid (all-*trans* RA) was purchased from Sigma Chemical Co. (St. Louis, MO). 9-*cis* RA was obtained from Kuraray Co. (Okayama, Japan). Acyclic retinoid was supplied from Eisai Co, Tokyo. The retinoids were dissolved in a 99.5% ethanol (5 vol)-dimethyl sulfoxide (1 vol) mixture at the concentrations indicated. Hoechst 33258 was purchased from Flow Laboratories, Inc. (McLean, VA).

Treatment of cells with retinoids. The human hepatoma-derived cell line, HuH-7 (8) was maintained in RPMI 1640 supplemented with 1% fetal calf serum (FCS) and 2% lactoalbumin hydrolysates. In order to determine the effect of the retinoids on cell growth, the cells were seeded at a density of 5×10^4 cells/ml per well with RPMI 1640 containing 1% FCS in Falcon 12-well plastic plates. After 24 hr, the medium was removed and the cells were washed three times with Hank's solution. After washing, FCS and lactoalbumin-free RPMI 1640 was added to each well. The cells were cultured for 48 hr, and the conditioned medium was replaced with FCS and lactoalbumin-free RPMI 1640 containing each retinoid. The number of viable cells was counted by the trypan-blue dye exclusion method.

DNA fragmentation assay. After retinoid treatment, the detached and the loosely attached cells were collected by flushing with phosphate buffered saline and the cell suspension was centrifuged at 400 × g for 10 min. The pelleted cells were lysed with STE (10 mM Tris-HCl pH 8.0, 25 mM EDTA, and 0.1 M NaCl) containing 0.5% SDS and 0.1 mg/ml of Proteinase K (Boehringer Mannheim) at 50°C overnight. The DNA was extracted twice with a solvent mixture of phenol:chloroform:isoamyl alcohol (25:24:1=vol/vol/vol). The DNA present in the aqueous phase was precipitated with 0.3 M Na acetate (pH 5.3) and 10 mM $MgCl_2$ and two volumes of ethanol at -20°C overnight. The pellet was washed with 70% ethanol and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The resultant DNA solution was loaded onto a 1.0% agarose gel in 1 × TBE (89 mM Tris, 80 mM boric acid, 0.2 mM EDTA pH 8.0). The fragmented DNA was visualized with ethidium bromide staining after electrophoresis (100 V, 1.5 hr).

Fluorochrome staining. The cells were treated with 5 μM of the different retinoids on glass tissue culture chamber/slides (Nunc, Naperville, IL) pre-coated with poly-L-lysine, fixed with Carnoy's solutions and then stained for 30 min with Hoechst 33258 fluorochrome. Photographs were taken with a Zeiss Axioskop camera microscope.

Results

Figure 1A shows the growth curve of the HuH-7 cells after retinoid treatment. The number of control cells remained constant until 24 hr after replacement by fresh FCS-free medium. After 36 hr, the cells grew exponentially up until the end of the experiment. Neither all-*trans* RA nor 9-*cis* RA changed the rate of cell growth during

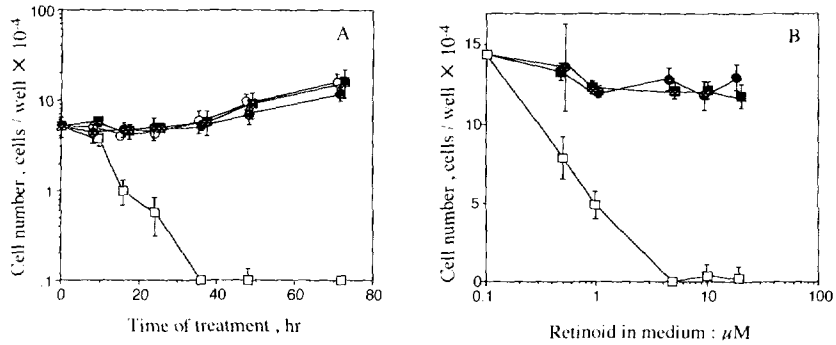

Fig. 1. Effects of acyclic retinoid on cell survival of HuH-7 cells in FCS-free medium.
(A) Time course of cytotoxicity induced by acyclic retinoid. The cells were treated with 5 μM of the different retinoids (● ; all-*trans* RA, ■ ; 9-*cis* RA, □ ; acyclic retinoid) or with the vehicle alone (○ ; control) in FCS-free medium. Viable cells were counted by the trypan-blue dye exclusion method at the indicated time points. Each point represents the mean ± S.E. (n=3).
(B) Concentration dependence of cytotoxicity induced by acyclic retinoid. Viable cells were counted after a 16-hr treatment with the different retinoids (● ; all-*trans* RA, ■ ; 9-*cis* RA, □ ; acyclic retinoid) at the indicated concentrations in FCS-free medium. Each point represents the mean ± S.E. (n=3).

the experimental period, even at the relatively high concentration of 5 μM. On the other hand, at the same concentration of acyclic retinoid, the number of viable cells started to decrease after 8 hr of treatment and very few cells were found attached after 16 hr of treatment. We next examined the range of concentrations of acyclic retinoid which can produce this cytotoxic effect. Viable cells were counted at 16 hr after retinoid treatment. Both all-*trans* RA and 9-*cis* RA again did not elicit any cytotoxicity at concentrations up to 20 μM, as shown in Fig. 1B. In contrast to these retinoids, acyclic retinoid exerted a significant cytotoxic effect. The cell number was reduced even at 0.5 μM acyclic retinoid and almost all of the cells were killed with concentrations greater than 5 μM,

To discriminate apoptotic cell death from necrotic cell death, we examined the biochemical features and morphologic changes of the cells after retinoid treatment. A 180-bp step-wise DNA fragmentation pattern was observed in the acyclic retinoid-treated cells at 8 hr after treatment, but no chromosomal DNA cleavage was detectable either in the all-*trans* RA or in the 9-*cis* RA treated cells at any time point (Fig. 2).

We were able to observe the characteristic chromatin condensation pattern of apoptotic cells when the acyclic retinoid-treated cells were stained with Hoechst 33258 (9), as shown in Fig. 3D. Such chromatin changes were not detectable in the all-*trans* RA- or 9-*cis* RA-treated cells. Hence, the acyclic retinoid-specific cytotoxic effects on the HuH-7 cells corresponded well to both the biochemical and morphologic features of apoptotic killing.

We found that the simultaneous addition of FCS with acyclic retinoid inhibited the retinoid-induced apoptotic cell death in a concentration-dependent manner (Fig. 4). With 0.5% FCS in the medium, the cell viability after acyclic retinoid treatment

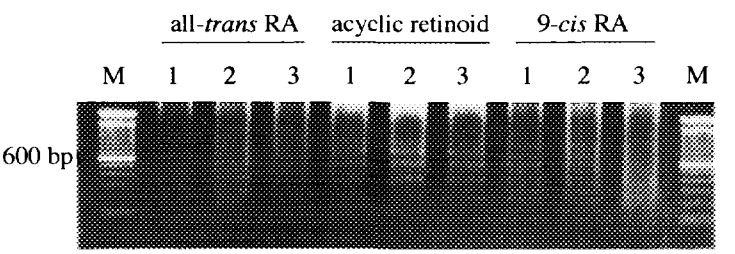

Fig. 2. Electrophoretic profiles of the genomic DNA from HuH-7 cells after treatment with the different retinoids.

Genomic DNA was extracted from the cells at the indicated times (1 : 8 hr, 2 : 16 hr, 3 : 24 hr) after treatment with 5 μM of the retinoids (all-*trans* RA, acyclic retinoid, 9-*cis* RA) and loaded onto a 1.0% agarose gel. The fragmented DNA was visualized with ethidium bromide staining. M, 100 bp DNA ladder marker (GIBCO BRL).

increased about 8-fold in comparison with the viability of the cells in FCS-free medium. In the presence of more than 2.0% FCS, acyclic retinoid, even at a concentration of 5 μM, could no longer induce cell death in the HuH-7 cells.

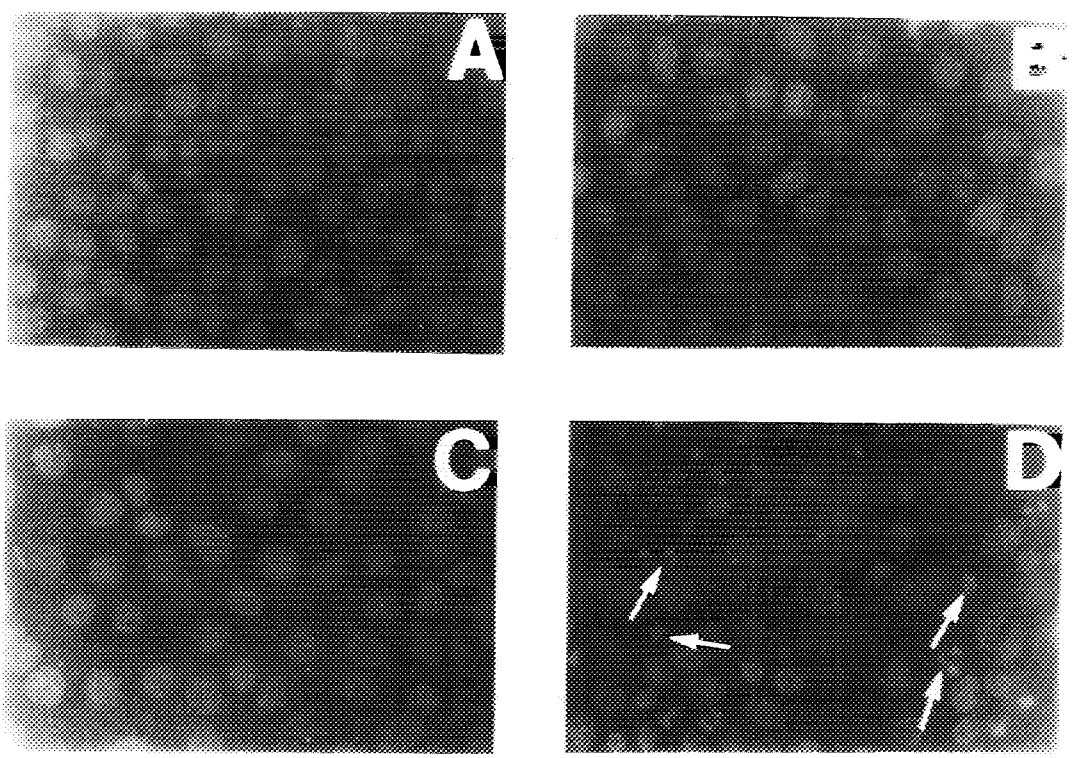

Fig. 3. Condensation of chromatin revealed by Hoechst 33258 staining.

The cells were treated with 5 μM of the different retinoids on tissue culture chamber slides precoated with poly-L-lysine, fixed with Carnoy's solutions and stained for 30 min with Hoechst 33258 fluorochrome. Arrows indicate the condensed chromatin in the acyclic retinoid-treated cells (panel D). In the control (panel A), all-*trans* RA (panel B) and 9-*cis* RA (panel C)-treated cells, no chromatin condensation was detectable.

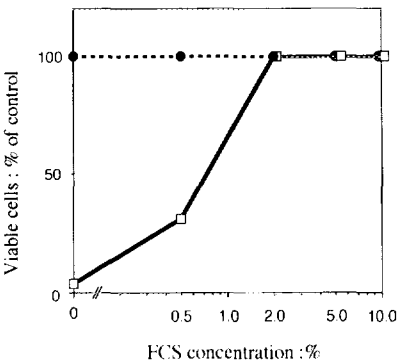

Fig. 4. Effects of serum concentration on acyclic retinoid-induced apoptosis in HuH-7 cells.
In the presence of the indicated concentrations of FCS, the cells were treated with (□) or without (● , control) 5 μM acyclic retinoid. Viable cells were counted 16 hr after the addition of acyclic retinoid. Each point represents the mean ± S.E. (n=3).

Discussion

This study clearly demonstrates that among the three retinoids tested, only acyclic retinoid at micromolar concentrations was able to induce apoptotic cell death in human hepatoma-derived HuH-7 cells. This was demonstrated unequivocally by the internucleosomal cleavage of the genomic DNA and the segmented condensation of the chromatin.

When we started this study, we thought that natural retinoids such as either all-*trans* RA or 9-*cis* RA would be able to induce apoptosis in HuH-7 cells, because retinoids have been known to play a key role in embryonal morphogenesis through apoptosis, and excess retinoid causes malformation by the induction of abnormal apoptotic death (2). Piacentini et al. (10) have reported that retinoic acid caused apoptotic cell death in human neuroblastoma cell lines. In fact, this report prompted us to pursue the experiments described in this study. We have shown that acyclic retinoid binds to CRABP (7) and also binds to the retinoic acid receptor (RAR) and thereby transactivates genes through the retinoic acid response element or binds to the retinoid X receptor (RXR) and transactivates genes via the retinoid X response element (Shidoji, Y. et al. submitted). However, these cytoplasmic and nuclear retinoid-binding proteins may not be involved in the induction of apoptosis, because no cell death occurred after treatment with all-*trans* RA, which is an endogenous ligand for both CRABP and RAR, or 9-*cis* RA, which is an endogenous ligand for RXR.

We recently have found that HuH-7 cells have enhanced transcription of the albumin gene and secrete more albumin into the medium upon treatment with acyclic retinoid (0.1 ~ 1.0 μM) without a significant change in the growth rate of the cells (11). In sharp contrast, here we found a drastic cytotoxic effect of acyclic retinoid on the same cell line. This apparent discrepancy can be explained easily by the serum-dependent inhibition of the cytotoxicity (Fig. 4). The previous experiment was conducted in the presence of 10% FCS in the medium, while in this work, the FCS was removed

from the medium 48 hr prior to treatment with the retinoids. This effect may not be unique for retinoid-induced apoptosis in HuH-7 cells, because another *in vitro* system, TGF-ß1 on Hep-3B cells, has been reported to require FCS-free medium for the induction of apoptosis (5).

As mentioned earlier, small lipophilic compounds, such as C_2-ceramide (3) and ether phospholipid (ET-18-OCH_3) (6), at micromolar concentrations have been shown to induce apoptotic death in HL-60 cells. In addition, polyunsaturated fatty acids are well known to alter carcinogenesis and have shown selective cytotoxic effects on human hepatoma cells in culture (12). These lipids are physiologic metabolites or their analogs so that acyclic retinoid may well mimic their effects on the induction of apoptosis, although the mechanism by which such a small lipid molecule can transduce an apoptotic signal has not yet been clarified.

The latency time for the cells to grow exponentially after medium replacement (Fig. 1A) may be explained by conditioning factors which are secreted by the cells into the medium and which are needed for growth. If so, we can not exclude the possibility that the acyclic retinoid may down-regulate the secretion of this putative growth factor(s) and that HuH-7 cells may be unable to survive without such factor(s). Thus, the retinoid-mediated metabolic withdrawal of this factor(s) may induce apoptosis.

Finally, we would like to discuss the possible significance of apoptosis in retinoid-mediated chemoprevention of cancer. In general, preneoplastic cells or highly proliferating cells are more susceptible to apoptosis than are normal or quiescent cells (13). It has been reported that the overexpression of the c-*myc* gene triggers apoptosis in cultured fibroblasts (14), although the c-*myc* gene is generally responsible for cell proliferation and not for growth suppression. We suggest that acyclic retinoid may eliminate existing preneoplastic cells via apoptotic cell death, resulting in cancer chemoprevention. A phase II clinical trial of acyclic retinoid as a chemopreventive agent for post-operative hepatoma is now in progress to test this hypothesis.

Acknowledgments

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