PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186**, 269–274 (1987)

Effects of Retinoid β-Glucuronides and *N*-Retinoyl Amines on the Differentiation of HL-60 Cells *in Vitro* (42612)

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Abstract. Retinoyl β-glucuronide and retinyl β-glucuronide, which are naturally occurring water-soluble metabolites of vitamin A, induce the granulocytic differentiation of HL-60 cells *in vitro*, as evidenced by an increased reduction of nitroblue tetrazolium. The relative effectiveness of various retinoids in differentiation is retinoic acid > retinoyl β-glucuronide > retinyl β-glucuronide. Under the selected assay conditions, retinol, hydroxyphenyl-retinamide, retinamide, and *N*-retinoyl-phenylalanine are essentially inactive in differentiation. At concentrations of retinoids from 10^{-9} to 10^{-5} *M*, cell viability was best with the retinoid β-glucuronides and retinamide, less with retinoic acid and retinol, and poorest with the *N*-retinoyl aromatic amines. Cellular growth was depressed only slightly by retinyl β-glucuronide and retinamide, but to a greater degree by the other derivatives. Retinoyl β-glucuronide was hydrolyzed in part to retinoic acid, whereas retinyl β-glucuronide was cleaved to retinol, if at all, at a very slow rate. Under the selected assay conditions, retinoic acid and the retinoid β-glucuronides primarily induce the differentiation of HL-60 cells, whereas the *N*-retinoyl aromatic amines show cytotoxicity.

In the presence of 10^{-6} *M* or lower concentrations of all-*trans* retinoic acid, the human promyelocytic leukemia cell line HL-60 differentiates into a granulocytic-type cell (1), which is associated with the reduction of nitroblue tetrazolium (NBT), with the expression of NAD-glycohydrolase (2), tissue transglutaminase (3, 4), cyclic AMP-dependent and calcium- and phospholipid-dependent phosphokinases (5, 6), as well as with other biochemical and morphological changes. While 13-*cis* retinoic acid was equally effective in inducing the differentiation of HL-60 cells, retinol, retinal, and retinyl acetate were much less active (1).

Two other types of retinoids are of interest in this regard, the naturally occurring retinoyl and retinyl β-glucuronides (7, 8) and a series of synthetic *N*-retinoyl amines that show chemopreventive properties against some forms of cancer (9). Retinoyl and retinyl β-glucuronides are formed in the liver, intestine, and other tissues, are secreted in the bile, are endogenous components of human plasma, are involved in an enterohepatic circulation, and show high activity in rat growth assays (7, 8, 10–13). Retinoyl β-glucuronide also stimulates the differentiation of the vaginal epithelium (14). Of the *N*-retinoyl amines, hydroxyphenyl-retinamide is the most efficacious chemopreventive agent against mammary cancer and is also highly effective against bladder cancer (9). Thus, these retinoids and related analogs were tested in the HL-60 cell system for their effects on differentiation, cellular viability, and cellular growth.

Materials and Methods. *Materials.* All-*trans* retinoic acid, all-*trans* retinol, nitroblue tetrazolium, insulin (from bovine pancreas), transferrin (human), and sodium selenite were obtained from Sigma Chemical Co. (St. Louis, MO); RPMI powdered medium was from GIBCO Laboratories (Grand Island, NY); and Corning plastic tissue culture flasks were from Fischer Scientific Co., (Itasca, IL). Retinyl β-glucuronide was synthesized from retinol (15), while retinoyl β-glucuronide (16), hydroxyphenyl-retinamide (17), *N*-retinoyl-phenylalanine (17), and retinamide (17) were synthesized by use of retinoyl-fluoride (18). All retinoids were analyzed for purity by reverse-phase gradient HPLC methods (16, 19).

Cell culture. Cultures of HL-60 cells were generously provided to us by Dr. Peter J. A. Davies, Department of Pharmacology, University of Texas Medical School at Houston. The cells originated from a culture obtained from the laboratories of Dr. R. C. Gallo, Na-

0037-9727/87 $1.50

tional Cancer Institute, National Institutes of Health (Bethesda, MD). Cells were grown in suspension in a defined, serum-free RPMI 1640 medium (3, 20) containing insulin (5 mg/liter), transferrin (5 mg/liter), and selenium (3 nM). The cells were cultured in plastic tissue culture flasks at 37°C in a humidified atmosphere of 7% CO_2. All retinoids were administered in 100% ethanol to flasks containing 4×10^5 cells in 5 ml of medium. Some of the less polar retinoids, e.g., retinol and retinamide, may not have been fully in solution at higher concentrations ($\geq 10^{-6}$ M). The final ethanol concentration (< 0.06%) had no apparent adverse effect. Flasks were incubated as indicated above for periods up to 72 hr. Cells were counted in a hemocytometer, and viability was examined by the exclusion of 0.4% trypan blue.

Measurement of NBT reduction. The extent of NBT reduction was measured by a slight modification of a method already described (3, 21). Cells ($1–2.5 \times 10^5$/ml) were incubated for 35 min at 37°C in the defined medium containing 1 mg/ml NBT and 0.1 μg/ml tetradecanoylphorbol acetate. After incubation, cells were immediately transferred to a hemocytometer for observation. In each case, about 200 cells were examined for the presence or absence of formazan clusters (blue–black deposits arising from the reduction of NBT). The amount of NBT reduction was then expressed as the percentage of the total number of cells observed which contained the dark formazan granules. The viability of the cells was high (>90%) in all cases where differentiation was significant. Thus the percentages of total cells and live cells that are differentiated are approximately the same.

Extraction and analysis of retinoids. The cell culture fluid including cells (5 ml) was treated with methanol (5 ml), vortexed, and extracted twice with ethyl acetate (5–8 ml). The pooled acetate phase was evaporated under argon to dryness and taken up in 100 μl of 2-propanol. Samples were stored at −27°C until used for HPLC analysis. By use of a gradient, reverse-phase HPLC system (19) (Waters Resolve 5-μm C18 column; methanol:water (65:35 with 0.1% ammonium acetate) to methanol:tetrahydrofuran (50:50) in 25 min, flow rate 1 ml/min, detection at 335 nm), retinoids were separated and identified by their elution times relative to standard compounds.

Results. The effects of various retinoids on the differentiation, viability and growth of HL-60 cells are summarized in Fig. 1 and Table I. Retinoic acid was the most effective compound in differentiation, followed by retinoyl β-glucuronide and retinyl β-glucuronide. Although retinamide may also show slight activity, the other retinoids were inactive in this regard. Differentiation was expectedly accompanied by a decrease in the growth rate. While retinoic acid was cytotoxic at somewhat higher concentrations (ED_{50} of 1.6 μM), retinoid glucuronides were not up to 10 μM. Retinol, while a poor differentiating agent, was quite cytotoxic (ED_{50} of 3.5 μM). Whereas retinamide had little if any adverse effect up to 1 μM, the highest concentration tested, both aromatic amides

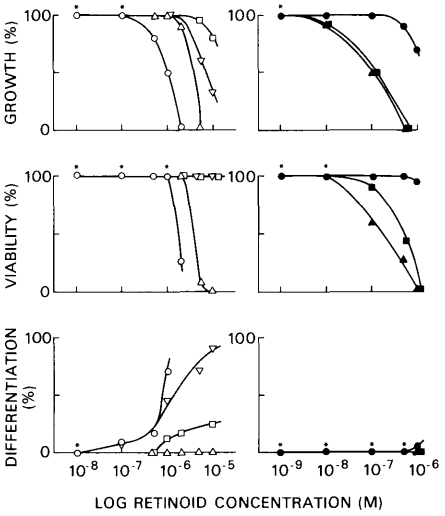

FIG. 1. Effects of all-*trans* retinoid concentrations on the percentage growth rate, viability, and differentiation of HL-60 cells, relative to control cells without retinoids present, in a 3-day incubation. Retinoic acid (○), retinoyl β-glucuronide (▽), retinyl β-glucuronide (□), retinol (△), retinamide (●), hydroxyphenyl-retinamide (▲), *N*-retinoyl-phenylalanine (■). Mean control cultures showed a 100% growth rate, 94% viability, and 3% differentiation. Points marked with an asterisk are common values for all retinoids tested.

TABLE I. THE 50% EFFECTIVE DOSE (ED_{50}) OF VARIOUS RETINOIDS ON THE DIFFERENTIATION, GROWTH, AND VIABILITY OF HL-60 CELLS

Retinoid	ED_{50}		
	Differentiation (μM)	Growth (μM)	Viability (μM)
Retinoic acid	0.8	1.0	1.6
Retinoyl β-glucuronide	1.4	3.5	>10
Retinyl β-glucuronide[a]	>10	>10	>10
Retinol	>10	3.5	3.5
Retinamide[b]	>1.0	>1.0	>1.0
Hydroxyphenyl-retinamide	ND[c]	0.10	0.10
N-retinoyl-phenylalanine	ND[c]	0.12	0.50

[a] Highest concentration tested, 10 μM.
[b] Highest concentration tested, 1 μM.
[c] Not detected.

were highly cytotoxic (Table I). With the latter two retinoids, cytotoxicity was time dependent; e.g., with 1 μM hydroxyphenyl-retinamide, 60% of the cells were dead at 18 hr, 85% at 36 hr, and >98% at 72 hr.

Major metabolites of the retinoids isolated after the 72-hr incubation are indicated in Table II and Fig. 2. Most esters of retinoic acid were hydrolyzed to some extent, being most evident with retinoyl β-glucuronide and least with *N*-retinoyl-phenylalanine. Retinol expectedly was largely converted to long chain fatty acyl esters. Interestingly, retinyl β-glucuronide was not cleaved at an appreciable rate; consequently, little or no retinol or retinyl ester was found in this case, and retinoic acid was not detected. Some nonpolar esters were noted when retinoic acid or its esters were added to the medium. Because significant destruction and isomerization of the added retinoids occurred during the 72-hr incubation, in all likelihood the polar compounds found in both control and experimental incubations are artifacts.

Discussion. Retinoic acid, either as all-*trans* or 13-*cis* isomers, markedly induces the granulocytic differentiation of HL-60 cells (1–6). Retinol, retinal, and retinyl acetate are much less active. We have confirmed these findings at retinoid concentrations similar to those employed earlier. Concurrently, the growth rate of the mixture of differentiated and undifferentiated cells decreases (1–3), an apparent expression of the general observation that differentiated cells divide more slowly, if at all, relative to their undifferentiated precursor cells.

The naturally occurring, water-soluble retinoid β-glucuronides have not previously been tested as differentiating agents. They are fairly active in this regard (Fig. 1 and Table I), however, while not showing cyto-

TABLE II. MAJOR METABOLITES OF RETINOIDS INCUBATED WITH HL-60 CELLS FOR 72 hr

Retinoid	Metabolites
Retinoic acid	Unidentified nonpolar esters
Retinoyl β-glucuronide	Retinoic acid, unidentified nonpolar esters
Retinyl β-glucuronide	Little or no retinol or retinyl ester, unidentified polar compounds
Retinol	Retinyl ester, unidentified polar compounds
Retinamide	Some retinoic acid
Hydroxyphenyl-retinamide	Some retinoic acid, unidentified nonpolar esters
N-retinoyl-phenylalanine	No retinoic acid, unidentified nonpolar esters

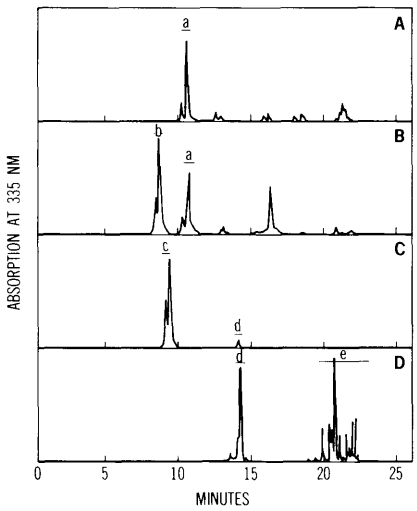

FIG. 2. Pattern of metabolities and other compounds found after 72-hr incubations of HL-60 cells with retinoic acid (A), retinoyl β-glucuronide (B), retinyl β-glucuronide (C), and retinol (D). Polar compounds are eluted early, nonpolar compounds late. Except for isomer formation, patterns have been corrected for polar artifacts appearing in controls with medium alone. Identified compounds are retinoic acid (a), retinoyl β-glucuronide (b), retinyl β-glucuronide (c), retinol (d), and retinyl esters (e). In all cases the absorbancy at the dividing crossbar (AUFS) is 0.032 OD units.

toxicity. Retinoic acid can initiate the differentiation of HL-60 cells at concentrations as low as 10^{-10} *M*, particularly in the presence of analogs of cyclic AMP (22) or by use of highly sensitive HL-60 cell sublines, such as the MRI strain (23). Thus, whether retinoyl β-glucuronide is active per se, or only serves as a precursor of retinoic acid, is not yet known. Interestingly, retinoic acid-binding protein, which seems to be required for the action of retinoic acid in some cell lines, has not been detected in HL-60 cells (24).

Retinyl β-glucuronide, on the other hand, is hydrolyzed very slowly, if at all, to retinol (Fig. 2). Although retinol is notably inactive in inducing the differentiation of HL-60 cells, retinol is extensively esterified to retinyl esters in these cells (Fig. 2). Retinol is not detectably converted to retinoic acid, however, either in our studies or in several embryonal carcinoma cell lines (25). Thus the distinct possibility exists that retinyl β-glucuronide is inducing HL-60 cell differentiation per se. Whether the retinoid β-glucuronides play a physiological role in cellular differentiation, either as water-soluble transport forms of vitamin A or as differentiating agents themselves, is not known. Because HL-60 cells differentiate in response to many agents, however, one clearly cannot generalize about cellular differentiation from the results obtained with any single cell line (25, 26). Nonetheless, the retinoid β-glucuronides merit further attention as differentiating agents (28).[1]

The apparent lack of toxicity of the retinoid glucuronides is also of interest. One of the greatest concerns is using retinoids as chemopreventive and therapeutic agents against cancer is their toxicity at or near their most effective dose (27). If, indeed, the retinoid glucuronides prove to be effective agents in this context, their low toxicity may well allow their use under conditions where other retinoids are contraindicated. In our research, Balb/c 3T3 mouse embryos cells as well as HL-60 cells were not adversely affected by the retinoid β-glucuronides up to 10 μ*M*.

The *N*-retinoyl amides may well act differently from retinoic acid and the retinoid glucuronides. Although retinamide had little effect, the two aromatic amides hydroxyphenyl-retinamide and N-retinoyl-phenylalanine were extremely cytotoxic and did not enhance differentiation under our culture conditions. The observed inhibition of cellular growth in these cases, therefore, can be attributed primarily to their toxicity. In contrast, Fontana *et al.* (29) observed that 1 μ*M* hydroxyphenyl-retinamide induced the differentiation of HL-60 cells, as indicated by the appearance of the OKM-1 surface antigen, when the cells were cultured in a medium containing fetal bovine serum. In their

[1] Our preliminary work appeared as an abstract (Fed Proc, Fed Amer Soc Exp Biol **46**:1187, 1987). M. Zile of Michigan State University and her colleagues have independently and concomitantly shown that retinoyl β-glucuronide induces the differentiation of HL-60 cells *in vitro* (28). Thus, our two studies are confirmatory in that regard.

studies all-*trans* retinoic acid was the most active retinoid tested, with high activity up to 10^{-5} *M*. Thus retinoic acid, when bound to proteins such as serum albumin, might well be less cytotoxic. Protein binding might also enhance or reduce its activity as an inducer of differentiation.

This research was supported by the U.S. National Institutes of Health (AM32793), the U.S. Department of Agriculture (CRG: 84-CRCR-1-1418), and the Allen Whitfield Memorial Cancer Fund. This is journal paper No. J-12399 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, and Project 2534.

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Received February 4, 1987. P.S.E.B.M. 1987, Vol. 186.
Accepted July 17, 1987.