Differential Binding to Soluble Nuclear Receptors and Effects on Cell Viability of Retinol and Retinoic Acid in Cultured Retinoblastoma Cells

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Summary: Human retinoblastoma cells in culture contain soluble receptors for both ^{3}H-retinol and ^{3}H-retinoic acid which are separate and distinct as assessed by specificity, enzyme susceptibility and binding affinity. Total cellular binding of retinol correlates well with its rapid effect on cell mortality. A limited number of soluble nuclear receptor sites for ^{3}H-retinoic acid but not ^{3}H-retinol are observed after incubation of the ^{3}H-retinoid with intact cells indicating a possible preferential effect of retinoic acid at the gene level.

Vitamin A has been well known to play a critical role in epithelial cell differentiation although the mechanism of action of retinol or retinoic acid is poorly understood (1). It also appears that retinol, retinoic acid and their analogs, collectively called "retinoids", are able to prevent or reverse the development of certain types of cancer (2). Recently, specific receptors for retinoic acid have been observed in several animal and human tumors (3,4).

Retinoblastoma is a hereditary disease of the retina (a neuroepithelial tissue) and is perhaps of viral etiology (5). Since a well-defined cell line of retinoblastoma (6) was available, we though it important to investigate the possible presence of vitamin A receptors in the cells and determine the effects of retinol and retinoic acid, the two major natural retinoids, on cell viability. The present communication 1) demonstrates the presence of such receptors in cultured human cells 2) compares the acute effects of the two retinoids on cell viability 3) demonstrates differential nuclear uptake of these retinoids and 4) establishes a system for detailed examination of the effects of retinoid

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analogs and for the study of the mechanisms of action of vitamin A in an established human cell line.

Methods: The cultured human retinoblastoma cell line (Y-79) has been previously described (6). Nuclei were prepared by the discontinuous sucrose gradient methods of Zieve (7); nuclear integrity was assessed after staining with hematoxylin. Sucrose gradient techniques were performed as previously described (8); protein was determined by the method of Lowry et al (9). ^{3}H-Retinol (2.45 Ci/mmol) was purchased from New England Nuclear Corp.; ^{3}H-retinoic acid (11.4 Ci/mmol) was a kind gift of Dr. W. Scott, Hoffmann-LaRoche, Nutley, N.J. All manipulations with the retinoids were either in the dark or under dim red light. In studies on the effects of enzymes on binding activity , supernatant samples were incubated for 5 min at 37°C with 1.2 units Protease (Type VI, S. griseus), 100 units phospholipase C, 50 g neuraminidase or with no added enzyme. Samples were then incubated with 2.8×10^{-7} M ^{3}H-retinol or 2.3×10^{-8} M ^{3}H-retinoic acid for 2 hrs at 4°C and subjected to gradient analysis. Enzymes were from Sigma Chem. Co.

Results: The 110,000 xg supernatant fraction of retinoblastoma cells readily binds ^{3}H-retinol, demonstrating a 2S receptor peak in sucrose density gradients (Fig. 1A). Binding is 33.7 pmoles of ^{3}H-retinol/mg supernatant protein under these conditions; this appears to be at least ten-fold higher than other tissues, including retina (10,11). Binding is relatively specific and reversible since non-radiolabeled retinol (200-fold excess) competes with ^{3}H-retinol for receptor binding and effectively dilutes out the 2S ^{3}H-retinol peak. The retinyl acetate ester also competes for receptor binding but retinyl palmitate and retinoic acid have no such effect. A similar binding pattern is observed when intact retinoblastoma cells are incubated with 1 μM ^{3}H-retinol for 30 min, washed, homogenized and the 110,000 xg supernatant fraction subjected to sucrose gradient analysis (Fig. 1B). Adding 200 μM non-radiolabeled retinol during the incubation period effectively dilutes out the ^{3}H-retinol binding peak.

A separate and distinct 2S receptor for ^{3}H-retinoic acid is also present in retinoblastoma supernatant (Fig. 1C). Unlabeled retinoic acid but not retinol competes effectively for receptor binding sites. ^{3}H-Retinoic acid uptake and binding in intact cells is also substantial (Fig. 1D). Addition of non-radiolabeled retinoic acid during the

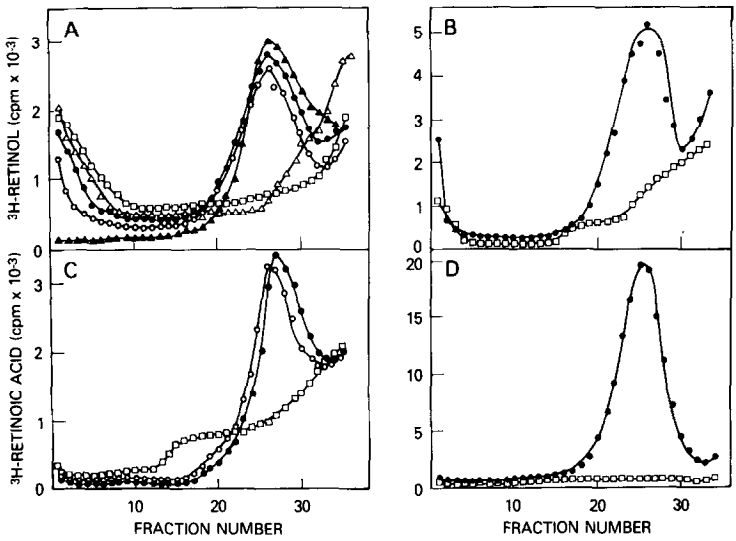

Figure 1. Sucrose density gradient centrifugation patterns of ^{3}H-retinol and ^{3}H-retinoic acid binding. Retinoblastoma cells were harvested by centrifugation at 100 xg, washed twice in buffered saline and homogenized in 10 mM tris buffer, pH 7.6, containing 1 mM disodium EDTA, 10 mM KCl and 1 mM dithiothreitol. Supernatant fractions were prepared by centrifugation at 110,000 xg for 45 min. When appropriate, 0.25 ml samples of total cell or purified nuclear supernatant was subjected to sucrose density gradient centrifugation (5-20% sucrose) as previously described (8). (A) - Incubation of retinoblastoma supernatant with 2.8×10^{-7} M ^{3}H-retinol alone (●—●) or in the presence of 5.6×10^{-5} M nonradiolabeled retinol, (△—△), retinyl acetate (□—□), retinyl palmitate (▲—▲) or retinoic acid (○—○) for 2 hrs at 4°C prior to gradient analysis. (B) Incubation of intact cells with 1×10^{-6} M ^{3}H-retinol alone (●—●) or in the presence of 2×10^{-4} M nonradiolabeled retinol(□---□) for 30 min at 37°C with subsequent preparation of the supernatant fraction and gradient analysis. (C) Incubation of supernatant with 2.3×10^{-6} M ^{3}H-retinoic acid alone (●—●) or in the presence of 4.6×10^{-6} M nonradiolabeled retinoic acid (□—□) or retinol (○—○) for 2 hrs at 4°C prior to gradient analysis. (D) Incubation of intact cells with 1×10^{-6} M ^{3}H-retinoic acid alone (●—●) or in the presence of 2×10^{-4} M nonradiolabeled retinoic acid (□—□) for 30 min at 37°C with subsequent preparation of the supernatant fraction and gradient analysis.

incubation period dilutes out the 2S peak with virtually no free, non-bound ^{3}H-retinoic acid in the supernatant, again indicating the availability of only a limited number of binding sites in the intact cell for this retinoid.

After incubation of the intact cell with 1 μM radiolabeled retinoic

Table I - Acute Effects of Retinol and Retinoic Acid on Retinoblastoma Cell Viability

Drug Concentration (μM)	Cell Viability (%)	
	Retinol	Retinoic acid
0	100	100
1	65	100
5	42	87
10	6	83
50	0	82
100	0	4

Cultured retinoblastoma cells were maintained in HEPES-buffered RPMI-1640 medium with 10% fetal calf serum in a 37° humidified incubator (6). The medium was changed every 3 days by decanting off half the spent fluid and then adding an equal volume of fresh medium. To determine the cytotoxicity of the retinoids, cells were first treated with 0.25% trypsin in calcium-magnesium free Dulbecco's buffered saline solution for 10 minutes. Aliquots were then placed in triplicate or quadruplicate petri dishes for each dosage level. Retinoids were dissolved in 95% ethanol at appropriate concentrations and subsequently diluted one-hundred fold in medium to obtain test solutions. Control medium contained 1% ethanol. Under such control conditions, at least 95% of the cells remained viable. The test solutions were added to the Y-79 cells and after 24 hours the cells were harvested, trypsinized and counted in a hemacytometer utilizing the trypan blue exclusion method for determining cell viability. Values given are averages of triplicate samples which agreed with 10%. Similar results were observed in two other experiments.

acid, 3-fold more ^{3}H-retinol than ^{3}H-retinoic acid is bound in the 2S peak (45 vs 14 pmoles/mg supernatant protein respectively). Retinol is also more toxic to the retinoblastoma cells (Table I). In acute 24 hour experiments, cell viability is only 65% of the control value with 1 μM retinol and 6% at 10 μM. Ten-fold higher concentrations of retinoic acid are needed to effect comparable cell death.

Differences between ^{3}H-retinol and ^{3}H-retinoic acid binding to receptors are also seen with enzyme treatment (results not shown). Pronase only decreases ^{3}H-retinol binding by about 30% but totally abolishes ^{3}H-retinoic acid binding in retinoblastoma supernatant. Phospholipase

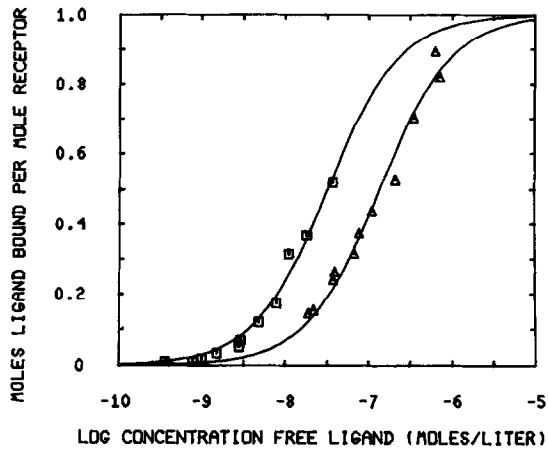

Figure 2. Computer plot of ^{3}H-retinol (△—△) and ^{3}H-retinoic acid (□—□) binding as a function of the logarithm of non-receptor bound ligand. Experimental conditions given in text. Data were analyzed by non-linear, least-squares curve fitting using the MLAB interpretative system and a DEC-10 computer exactly as previously descirbed (12).

C has a small (20% decrease) but definite effect on only retinoic acid binding while neuraminidase has virtually no effect on either ^{3}H-retinol or ^{3}H-retinoic acid binding. Binding data also indicate substantial differences between ^{3}H-retinol and ^{3}H-retinoic acid binding (Fig. 2). Computer analysis by the logarithmic plot (Bjerrum's formation) as previously described (12) yields dissociation constants (K_D) of 1.4×10^{-7} and 3.2×10^{-8} for retinol and retinoic acid respectively indicating relatively high but distinctly different (23-fold) affinity values for the two retinoids.

The most significant differences are observed however in retinol and ^{3}H-retinoic acid uptake and binding in retinoblastoma nuclei. When nuclei are isolated, the nuclear supernatant fraction prepared and then incubated with ^{3}H-retinol or ^{3}H-retinoic acid, no specific binding of either retinoid is observed (Fig. 3A and B). Similarly, when intact cells are first incubated with radiolabeled retinoid (1 μM as described for Fig. 1B and D) and the nuclei subsequently isolated, no specific binding

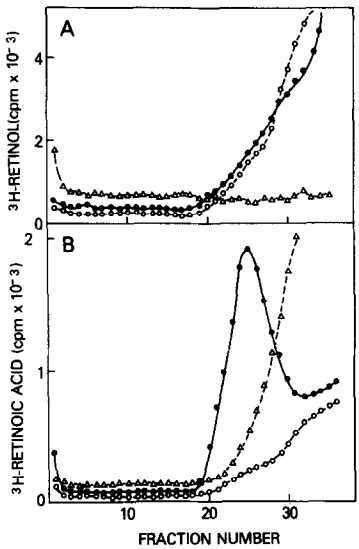

Figure 3. Sucrose density gradient centrifugation patterns of retinoblastoma nuclear supernatant. (A) Nuclear supernatant prepared as in Fig. 1 and incubated wtih 2.8 x 10^{-7}M ^{3}H-retinol for 2 hrs at 4°C prior to gradient analysis (△—△). Nuclear supernatant prepared after intact cells were incubated with 1 x 10^{-6}M ^{3}H-retinol for 1 hr at 37°C in the absence (●—●) or presence (○—○) of 2 x 10^{-4}M nonradiolabeled retinol and subsequently subjected to gradient analysis. (B) Nuclear supernatant incubation with 2.3 x 10^{-8}M ^{3}H-retinoic acid for 2 hrs at 4°C prior to gradient analysis (△—△). Nuclear supernatant prepared after intact cells were incubated with 1 x 10^{-6}M ^{3}H-retinoic acid for 1 hr at 37°C in the absence (●—●) or presence (○—○) of 2 x 10^{-4}M nonradiolabeled retinoic acid and subsequently subjected to gradient analysis.

of ^{3}H-retinol is detectable in the nuclear soluble fraction. In contrast, a distinct 2S peak of bound ^{3}H-retinoic acid is observed in the soluble fraction of nuclei isolated after preincubation of intact cells with ^{3}H-retinoic acid (6.4 pmoles ^{3}H-retinoic acid bound/mg of nuclear soluble protein). Binding of ^{3}H-retinoic acid to the nuclear receptor is specific and saturable since a 200-fold excess of non-radiolabeled retinoic acid present during the initial incubation totally abolishes the 2S ^{3}H-retinoic acid peak.

Discussion: Retinol receptors are present in several normal cell types (10-14). Retinoic acid receptors have only recently been demonstrated in normal (10,14,15) and tumor (3,4) tissue and have now been reported

in the nuclei of some tissues (16). From the present study, it appears that separate and distinct receptors for retinol and retinoic acid are present in retinoblastoma cell supernatant with both receptors sedimenting at 2S. No 7-8S retinol receptor species is apparent in this cell line, however, as previously shown to be present in normal human retina (11). Since no photoreceptor membranes are present in the retinoblastoma cells, these findings are consistent with the possibility that the 7-8S receptor is compartmentalized in retinal photoreceptor units (11). The present study also demonstrates that not only can specific binding be observed in supernatant preparations in vitro but also that substantial and specific uptake and binding can be demonstrated after incubation with intact cells. There appears to be good correlation between the high uptake and binding of retinol and its rapid effect on cell mortality over an acute 24 hour culture period. Retinoic acid has a much less pronounced effect on cell mortality but appears to be preferentially taken up and bound in retinoblastoma nuclei. Although binding to insoluble nuclear components has yet to be examined, this system may prove useful in the future as a model for differentiating the effects of retinoid analogs on general cell growth characteristics (e.g. cell division) and on viability.

It is particularly interesting that neither ^{3}H-retinol nor ^{3}H-retinoic acid receptors are observed when isolated nuclei are homogenized and the supernatant fraction incubated with the retinoids. ^{3}H-retinoic acid (but not ^{3}H-retinol) is bound to soluble nuclear receptors only after the retinoid is incubated with intact cells. This situation is similar in many ways to that seen with steroid hormone receptors where nuclear binding is only detectable after hormone interaction with cytoplasmic receptors (17) and is different from results of a recent study which found both a 2S ^{3}H-retinoic acid binding-protein and a 5S albumin-like binding component in the 0.3M KCl extract of nuclei isolated prior to incubation with ^{3}H-retinoic acid (16).

It is thus clear that uptake and binding of ^{3}H-retinol is different from that of ^{3}H-retinoic acid at least in human retinoblastoma cells. The presence of soluble nuclear receptors for ^{3}H-retinoic acid but not for ^{3}H-retinol may indicate separate roles for the two retinoids in cells with retinoic acid possibly involved in events at the gene level.

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