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INDUCTION OF DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS BY RETINOL: POSSIBLE MECHANISMS

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Retinol, like retinoic acid, can induce differentiation of two embryonal carcinoma cell lines. These cells contain the specific retinol binding proteins in the cytosolic fractions. No correlation was found between the levels of binding activity and the effectiveness of retinol to induce differentiation. Microsomal fractions of embryonal carcinoma cells catalyse mannosyl transfer from GDP-mannose to retinyl phosphate. The formation of mannosyl retinyl phosphate appears a constitutive property and can be a mechanism by which retinoids induce early cell surface changes during differentiation of embryonal carcinoma cells.

Retinoids (vitamin A analogs) can induce differentiation in several cell types in vitro. These compounds stimulate differentiation of various embryonal carcinoma (1, 2), promyelocytic leukemia (3) and mammary cell lines (4). The mechanism by which retinoids act is, however, not yet elucidated. Chytil and Ong (5) have proposed that these compounds may alter transcription via the mediation of specific retinoid binding proteins. An alternative was suggested by De Luca and Wolf (6, 7) implicating phosphorylated intermediates of retinoids in glycosyl transfer reactions to endogenous acceptors. In this way retinoids could alter specific glycoconjugates and affect biological processes such as differentiation. Recent studies by Bolmer and Wolf (8) on the release of fibronectin in enucleated cells show

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that certain actions of retinoids don't require a nuclear involvement. Rizzino (9) has reported recently that embryonal carcinoma cells can differentiate in the absence of retinoids indicating that retinoids are not an absolute requirement.

Several studies have shown the importance of cell surface characteristics in the differentiation of embryonal carcinoma cells. Hogan et al., (10) reported that the extracellular matrix can direct differentiation into visceral or parietal endoderm. Ogiso et al., (11) have shown that retinoids induce the disappearance of peanut agglutinin receptors in an early phase, a change that appeared reversible after removal of retinoic acid. These results suggest a role for cell surface changes in the induction of differentiation of embryonal carcinoma cells.

In this paper we demonstrate that microsomes of embryonal carcinoma cells can catalyse the synthesis of mannosyl retinylphosphate. The formation of this compound may be involved in the early cell surface changes during differentiation.

MATERIALS AND METHODS

HL60 cells were obtained from Dr. T. Breitman (NCI, Bethesda). PCC4 aza 1R2 and F9 embryonal carcinoma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Induction of differentiation was measured as described previously (2, 12). Binding proteins were assayed as described previously (13) via polyacrylamide gel electrophoresis which has been proven to be more sensitive than determination via sucrose gradient (14). Binding was carried out at 10^{-7} M which gives maximum binding. ^{3}H-retinoic acid (40 Ci/mmol) was a gift from Dr. W. E. Scott (Hoffmann LaRoche, Nutley, N.J.) ^{3}H-retinol (36 Ci/mmol) was obtained from Amersham. The synthesis of mannosyl retinylphosphate was determined as described previously (15). Briefly, the standard incubation mixture contained 0.1 µCi GDP-[^{14}C]-mannose, 136 µM retinyl phosphate, 0.8 mg bovine serum albumin, 24 µM GDP-D-mannose, 30 mM or Tris-HCL (pH 8.0) 5 µM $MnCl_2$, 8 mM or NaF, 2 mM or ATP, 5 mM or AMP and 0.7 mg of crude membrane preparation in a total volume of 200 µl. After incubation reaction was stopped by the addition of 1 ml ice-cold buffer and immediately filtered on Millipore filters (0.45 µm). Filters were washed with buffer and radioactivity determined in hydrofluor. For identification radioactive compounds were extracted with 1 ml chloroform/methanol (2:1) and thin layer chromatography carried out with the lower phases using chloroform/methanol/water (45:35:6, by vol.) as solvent. Fluorography was carried out on Kodak XR-5 film.

RESULTS AND DISCUSSION

Both retinol as well as retinoic acid are able to induce differentiation of embryonal carcinoma cells. However, as shown in Table 1, reti-

TABLE 1. Comparison of the induction of differentiation by retinoids and the levels of retinol and retinoic acid binding proteins.

Cell line	Minimum concentration to induce differentiation		CRBP	CRABP
	Retinoic acid (M)	Retinol (M)	(f moles/mg protein)	
PCC4aza 1R2	10^{-8}	10^{-6}	2450	1750
F9	10^{-9}	10^{-7}	750	550
HL 60[1]	10^{-9}	10^{-7}	ND[2]	ND

Binding of retinoids was determined at 2.10^{-7} M, what has been shown to give optimum binding (17).
[1]From ref. 3.
[2]ND = Not detectable

noic acid appears to be far more active than retinol. Both embryonal carcinoma cell lines tested contained the specific retinol and retinoic acid binding proteins (CRBP and CRABP, respectively) in the cytosolic fractions. Comparison between the binding protein levels and induction of differentiation shows that this biological activity does not correlate with the levels of either CRBP or CRABP. In monolayer culture, much higher concentrations of retinol or retinoic acid are needed to induce differentiation of PCC4aza 1R2 than for the induction of F9 cells whereas the levels of the binding proteins are much higher in the former cells. Another result that shows a poor correlation between binding proteins and biological activity is that retinol is much less active than retinoic acid whereas the levels of CRBP and CRABP are of the same magnitude. This difference in activity between retinol and retinoic acid maybe explained by rapid metabolism of retinol into metabolites unable to bind to CRBP or perhaps by the difference in hydrophobicity between the two molecules. Another question that remains unanswered is whether both CRBP as well as CRABP are important in the differentiation of embryonal carcinoma cells. It is possible that only CRABP is important and that the biological activity of retinol is due to the conversion of retinol to retinoic acid. In contrast to the results above that show a poor correlation between biological and binding activities, several other findings have suggested a relationship. The absence of CRABP in mutants that are unable to differentiate in the pre-

sence of retinoic acid (16) and the structural specificities of the binding and biological activities of retinoids (17) strongly support the idea that CRABP is involved in the induction of differentiation. It is striking that in monolayer cultures the PCC4aza 1R2 cells need much higher concentrations of retinoids to induce differentiation than when cells are grown as aggregates. This may indicate that other factors are involved in embryonal carcinoma differentiation. This idea is supported by Rizzino's findings demonstrating differentiation of embryonal carcinoma cells in the absence of retinoids (9). Comparison of the induction of differentiation of embryonal carcinoma cells with that of the promyelocytic leukemia cells HL60 shows that neither CRABP nor CRBP could be detected in the cytosolic fractions. The results on CRABP are in agreement with previous findings of Douer and Koessler (18). The absence of binding proteins in HL60 cytosolic fractions makes it necessary to search for alternative mechanisms of retinoid action.

Cell surface changes appear to play a role in embryonal carcinoma cell differentiation and retinoids have been shown to alter cell surface characteristics (19, 20). A mechanism by which retinoids could affect cell surface properties has been proposed previously (13) and involves the synthesis of glycosylated intermediates which could alter glycosylation processes. The best characterised intermediate is mannosyl-retinylphosphate (MRP)(15,21,22).

The time course of mannosyl transfer from GDP-mannose to microsomes is shown in Fig. 1. Liver microsomal preparations were used for comparison purposes since formation of MRP by liver microsomes has been well established (15) and the activity is the highest of several tissues tested (unpublished results). In the presence of 0.136 M retinylphosphate the transfer of mannose proceeded linearly for about 5 min then leveled off slowly, followed by a gradual decrease. In the absence of retinylphosphate very little radioactivity is transferred. All three cell lines tested contain this activity but mannosyl transfer was the lowest in HL60 preparations. The initial rate of mannosyl transfer was measured at 2 min and a

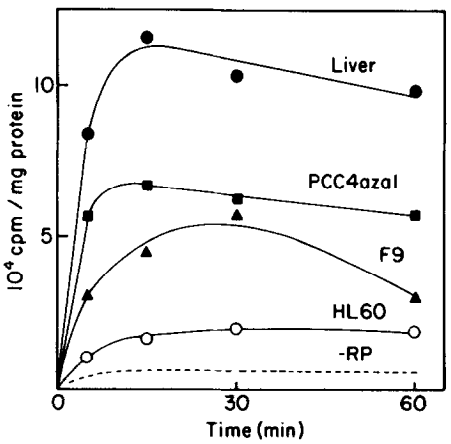

Figure 1. Time course of mannosyl transfer by crude membrane preparations of liver. PCC4aza 1R2, F9 and HL 60. Radioactivity retained on the filter in the presence (solid lines) or absence (dashed line) of retinyl phosphate was plotted against incubation time.

comparison shown in Table 2. The embryonal carcinoma cells PCC4 aza 1R2 and Fq show initial rates of 28.6 and 37.1% whereas HL60 microsomal fractions contain only 14.7% that of liver.

The labeled compounds were characterised via thin-layer chromatography (Fig.2). In the absence of retinylphosphate only one major band can be seen previously identified as dolichylmannosylphosphate (Rf = 0.9). In the presence of retinyl phosphate the major band corresponds to MRP and migrates as a doublet (Rf = 0.7). Several other bands can be seen, one at the origin consisting of mannose-1-phosphate, a degradation product of MRP. Furthermore, a few minor bands are visible which have not been identified but which seem to be dependent on the presence of retinyl phosphate.

TABLE 2. Comparison of the initial rate of MRP synthesis by membrane fractions of various cell types.

Cells	Synthesis of MRP (cpm/mg protein/2 min)	Percentage from liver
Liver	37434	100
PCC4aza 1R2	10705	28.6
F9	13872	37.1
HL 60	5516	14.7

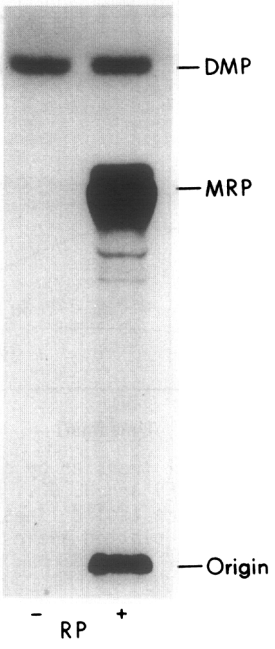

Figure 2. Fluorograph of thin-layer chromatography of labeled mannolipids synthesized by a crude membrane preparation of F9 cells. RP = retinylphosphate; DMP = dolichylmannosylphosphate; MRP = mannosylretinylphosphate.

In order to see whether the activity was constitutive or inducible, cells were grown in the absence of retinol for several days. Then to one half of the cells 10^{-7} M retinol was added and the mannosyl transfer reaction measured. As shown in Fig. 3 the transfer of mannose was very similar

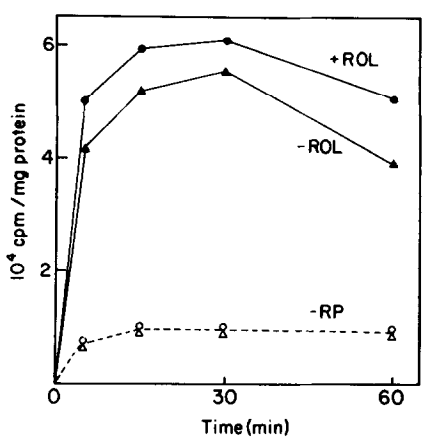

Figure 3. Time course of mannosyl transfer by crude membrane preparations of PCC4aza 1R2. Cells grown in vitamin A-free medium were grown in the presence and absence of 10^{-7} M retinol. Crude membrane preparations were isolated and mannosyl transfer determined in the presence (closed symbols) and absence (open symbols) of retinylphosphate.

in the microsomal preparation from cells grown in the presence or absence of retinol indicating the constitutive character of this enzymatic activity.

Similar intermediates as for retinol have been recently identified for a metabolite of retinoic acid (21). It has been shown that cell surface properties are important to direct the differentiation of embryonal carcinoma cells (10). Moreover, Ogiso et al., (11) have shown that retinoids can induce the disappearance of peanut agglutinin receptors in an early phase, and in a reversible manner. The synthesis of glycosylated intermediates of retinoid metabolites could be a mechanism by which retinoids induce the early cell surface changes observed during differentiation of embryonal carcinoma cells.

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