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13-CIS-RETINAL STIMULATES PROLIFERATION AND INDUCES INTRANUCLEAR PROTEIN ACCUMULATION IN THE HUMAN MAMMARY TUMOR CELLS MCF-7*

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Summary: The human mammary tumor cells MCF-7 show enhanced proliferation when treated with low dosis (10^{-8} - 10^{-7} M) of 13-cis Retinal (a vitamin A derivative). These results are independent of the growth medium used. We describe a novel effect of 13-cis Retinal: the increased synthesis and accumulation of nuclear proteins in cronically treated cells. The cytoplasmic proteins and proteins released to the culture medium are transiently and oppositly modified. Moreover, cronic treated cells have growth advantages over the untreated counterparts in a clonogenic soft agar assay.

Retinoids (vitamin A and its derivatives) have been proposed as cancer preventive agents since many human and rodent malignant cells cease growing when they are treated usually with milimolar concentrations of these compounds (1).

However, less successful clinical results were obtained in cancer patients, having been evoked, among others, pharmacological reasons for the failures of the clinical treatments (2,3,4). Also in tissue culture studies, the effects of retinoids on cellular proliferation depends not only on the cell type but on the components of the growth medium (5). Cells transformed by Kirsten and Moloney sarcoma virus are remarkably stimulated by retinoids when growing in a chemically defined medium while the addition of serum masked these effects (6). The authors suggest that the production of growth factors by these cells is a necessary, although not sufficient, condition for the stimulatory action of retinoids. Also, retinoic acid inhibits the anchorage-independent growth of the

* Dedicated to Professor Luis F. Leloir on the occasion of his 80th anniversary.
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ABBREVIATIONS: FCS: Fetal Calf Serum; DM: Chemically Defined Medium; Rds: Retinoids; Ral: (13-cis Retinal); DMSO: Dimethylsulfoxide; TCA: Trichloroacetic Acid; PBS: Phosphate Buffer Saline; S.E.M.: Standard Error of the Mean.

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Sirian hamster cells expressing v-src but enhances the anchorage-independent growth of the same cell line expressing v-Ha-ras (7). The human breast tumor cells are also an example of the contradictory results obtained with retinoids. While "in vitro" they are strongly inhibited by retinoic acid (8,9) no activity has been obtained in patients with metastatic breast cancer (10). In the present communication, we use the human mammary tumor cell line MCF-7, to study the effect of 13-cis Retinal. This cell line is able to grow in a chemically defined medium with the only requisite of insulin as a growth regulatory factor (11). When treated with 13-cis Retinal these cells show a marked stimulation of cellular proliferation either with FCS or in the chemically defined medium. The most striking observation is that cronically treated cells show a dramatic increase in the amount of ^{35}S-methionine incorporated into nuclear proteins with a concomitant increase in the nuclear protein mass, while the cytoplasmic and "secreted" proteins go through temporary and opposite modifications of rise and decrease. Retinal-treated cells show also the ability to grow as macrocolonies of loosely packed cells in a soft agar medium while untreated cells fail to grow in the same medium.

MATERIALS AND METHODS

Chemicals: 13-cis Retinal (2-cis-Vitamin A derivative) Type XV was purchased from Sigma Chemical, Co. (St. Louis, Mo.), dissolved in DMSO at 10^{-2} M for stock solution and stored at -70°C. All retinoids manipulations were done in subdued light.

Cell culture: The human uncloned mammary tumor cells MCF-7 were obtained and grown as previously described (11).

Cell growth experiments: Cellular proliferation studies were performed as follows: MCF-7 cells were plated in 35 mm dishes containing Dulbecco/F-12 (1:1) supplemented with insulin (10 ug/ml) and 10% FCS.4 hrs after plating, and when indicated, the dishes were extensively washed with medium lacking serum, and shifted into Dulbecco/F-12 plus insulin and 1 mg/ml BSA (FV, Sigma Co., St. Louis, Mo.). Cells were incubated at 37°C in a humidified atmosphere containinng 5% CO_2. Medium was changed every three days. Rd treatment was continuous and replenished on every occasion of medium change. Cell number was determined in duplicate using a haemocytometer. Viable cells were counted after trypan blue exclusion.

Agar assays: Agar bases (1 ml in 35 mm Petri dishes) were made with Dulbecco/F-12 medium supplemented with 10% FCS.1-2x10^3 cells were seeded into agar containing growth medium (0.4% agar) supplemented with 10% FCS and 10^{-8} M Ral. The cells were fed weekly with the same medium. After 2 weeks, 0.5 ml of a sterile solution of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride (0.5 mg/ml in water) was layered over the agar and the incubation was

continued for 24 hrs. Cells were photographed under a Wild microscope with a Polaroid film.

Protein synthesis and separation into nuclear and cytoplasmic fractions: Cells were labeled for 3 hrs with 100 uCi/35 mm plate with [^{35}S]-methionine (1000 Ci/nmole, New England Nuclear, Boston, MA) in medium lacking methionine. After incubation cells were washed twice with cold PBS, scrapped off with a rubber policeman, span down for 2 minutes in an Eppendorf centrifuge and resuspended in 50 ul of TNN Buffer at 0°C (50 mM Tris pH 8.0, 250 mM NaCl, 0.5% NP-40, 1 mM PMSF). Similar results were obtained by trypsinization although the amounts of cytoplasmic proteins were slightly lower in both control and cronically treated cells probably due to the protease action on membrane proteins. This suspension was kept on ice for 4 minutes with occasional vortexing after which it was homogenized with 10 strokes in a Dounce homogenizer and centrifuged for 2 minutes in Eppendorf to separate nuclei from the soluble fractions. Proteins were precipitated with 10% trichloroacetic acid, dissolved in 0.1 N NaOH and counted with Bray scintillation cocktail. Unlabeled samples were processed as above, except that extracts were used for protein determinations by Lowry's technique (12).

Trichloroacetic acid-soluble ^{35}S-methionine: Cells were labeled as above described; afterwards they were quickly washed with cold PBS and the soluble radioactivity extracted with 10% TCA and counted in Bray.

RESULTS

The effect of 13-cis Retinal on cellular proliferation was studied in cells growing in the presence of FCS or in the DM. The dose-response curves are shown in Fig. 1 (A and B). The optimal concentration of 13-cis retinal for growth stimulation was 10^{-8}-10^{-7} M, while 10^{-6} M produces growth inhibition which is always much more important 2-3 days after plating the cells. Afterwards, and in spite of the addition of fresh medium containing 13-cis Retinal every three days, cells seem to recover and are able to grow at similar rates as controls. Concentration of 13-cis Retinal above 10^{-6} M were toxic in all the experiments performed. The growth-stimulatory effect is almost identical in FCS or in the DM, while the cell's morphology does not show significant changes: cells remain with the epithelioid morphology in FCS or with the less differentiated one shown in the DM as previously described (11) (data not shown). Essentially, the same results were obtained with retinoic acid (data not shown). We examined the effect of 13-cis Retinal on the synthesis of cellular proteins as well as on proteins released to the culture medium. Table I shows the transient effect of the drug on the incorporation of ^{35}S-methionine and on the protein content of cells growing in medium containing FCS. While an acute treatment shows a slightly inhibition in the incorporation of the radioactive precursor into

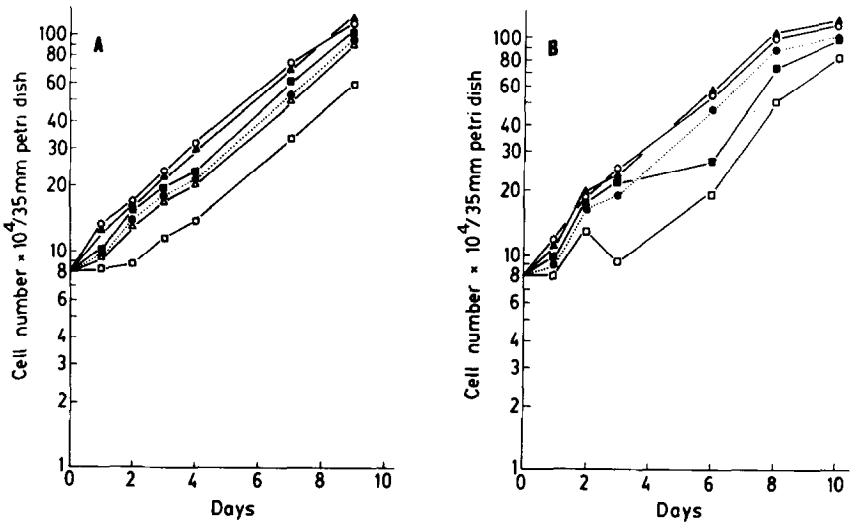


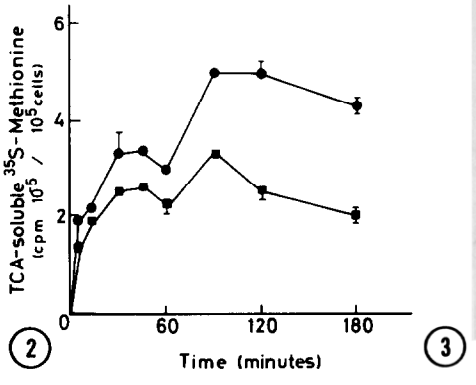
Figure 1. Dose-response curves of MCF-7 cells treated with 13-cis Retinal.
A) Cells growing with 10% FCS.
● Controls; △ Controls + 0.1% DMSO; □ 10^{-6}M Ral; ■ $5x10^{-7}$M Ral; ○ 10^{-7}M Ral; ▲ 10^{-8}M Ral.
B) Cells growing in a chemically defined medium (explicited in Materials and Methods). Symbols as in Fig. 1A. A representative experiment is shown. Similar experiments have been obtained in additional experiments.

intracellular proteins and in proteins released to the culture medium, after 15 days of treatment there is a 50-60% increase in the incorporation of ^{35}S-methionine and in the total protein content. The fractionation into nuclear and soluble fractions shows that both have increasing amount of radioactivity and in the protein mass. The protein products released to the culture medium are still slightly inhibited. A cronic 60 days' treatment shows a striking difference: while the soluble fraction now is very similar to controls, the nuclear fraction has almost three times the protein content and the radioactivity of the untreated cells, with a 40% increase in proteins released to the culture medium. The above results are even more dramatically modified after a 4 months' treatment. We want to stress that the increased incorporation of ^{35}S-methionine into TCA insoluble fractions of Ral-treated cells is not a consequence of an augmented transport of the radiolabeled aminoacid. On the contrary after 60 min incubation the amount of soluble radioactivity is higher in control than in Ral-treated cells (Fig. 2). Around 20-30% of the cronically treated cells growing with FCS when plated in a soft agar medium are able to

Table I. Effect of 13-cis Retinal on protein synthesis and protein accumulation in acute and cronic treatments with 13-cis Retinal

	Total cellular proteins				Cytoplasmic proteins				Nuclear proteins				Proteins released to the medium	
	Incorporation of ^{35}S-Meth.		Quantification by Lowry's technique		Incorporation of ^{35}S-Meth.		Quantification by Lowry's technique		Incorporation of ^{35}S-Meth.		Quantification by Lowry's technique		Incorporation of ^{35}S-Meth.	
Days of treatment	cpm x $10^{-5}/10^5$ cells	%	pgr/cell	%	cpm x $10^{-5}/10^5$ cells	%	pg/cell	%	cpm x $10^{-5}/10^5$ cells	%	pgr/cell	%	cpm x $10^{-3}/10^5$ cells	%
Controls	1.86	100 (18)	573	100 (7)	1.24	100 (18)	384	100 (7)	0.62	100	189	100 (7)	1.74	100
3 days	1.47	78 (4)	N.D.	-	N.D.	-	N.D.	-	N.D.	-	N.D.	-	1.54	89 (4)
15 days	2.92	157 (8)	860	150 (2)	1.86	150 (8)	550	143 (2)	1.00	162 (8)	310	164 (2)	1.34	77 (8)
60 days	3.05	164 (3)	951	166 (2)	1.30	105 (3)	409	106.5 (2)	1.80	292 (3)	542	287 (2)	2.40	138 (3)
110-120 days	4.70	253 (3)	1.400	244 (2)	2.52	203 (3)	640	167 (2)	2.58	419 (3)	720	381 (2)	1.41	81 (3)

Treated cells were grown with 10^{-8} M 13-cis Retinal for the times described. Brackets represent the number of different experiments, each performed in duplicate. Experimental details in Materials and Methods. N.D.: not determined.



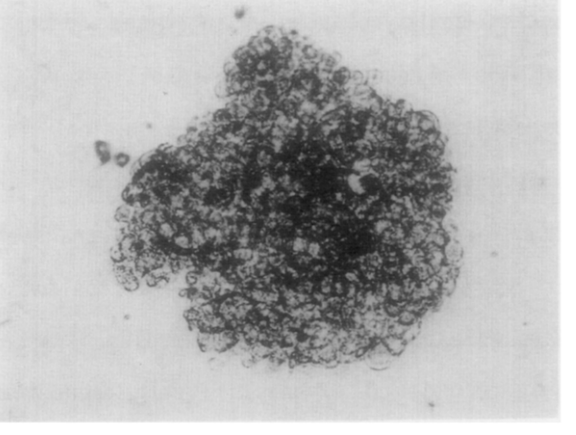


Figure 2. TCA-soluble ^{35}S-methionine of control and cronically treated cells. Cells were plated as for protein synthesis determination. The amount of soluble TCA was determined as described in Materials and Methods. ● controls; ■ Ral-treated cronic cells (110-120 days + 10^8 MRal). S.E.M. is indicated where it was larger than the symbols.

Figure 3. Agar-growing colony (>0.5 mm^2) of MCF-7 cells treated for 60 days with 10^{-8}M Ral. Experimental details in Materials and Methods (x 100).

grow as macrocolonies of loosely packed cells, while and as it was previously described (11) untreated cells fail to grow in the same medium (data not shown). Fig. 3 shows an example of the type of colonies obtained with 13-cis Retinal.

DISCUSSION

13-cis Retinal stimulates proliferation of MCF-7 cells in our case independently of the presence of bovine serum in the growth medium, since its action is very similar in cells growing in the DM (Figs. 1, A and B). It has been reported that MCF-7 cells produce EGF (13), TGF (14) and probably other as yet unknown growth factors (11). It has also been postulated that the mode of cellular transformation could modulate the response to retinoids probably through the secretion of transforming growth factors (6). Since, in our case, serum does not mask the proliferative response to 13-cis Retinal, the mode of action of retinoids appears to be of a very complex nature. The above results are not restricted to MCF-7 since the mammary tumor cell line from metastatic origin, T-47 c11 (15) and a cell line derived from a mammary carcinosarcoma, Hs578T (16) showed increased proliferation when treated with 13-cis Retinal or reinoic acid (data not shown). We also describe in this paper a novel effect of 13-cis Retinal: the nuclear protein accumulation in cronically treated cells. These results could reflect an increased synthesis and/or transport of the nuclear proteins although protein stabilization could also be considered. In this respect, the action of 13-cis Retinal is similar to the action of PDGF in quiescent 3T3 fibroblasts (17) in which it also induces intranuclear protein accumulation although probably as a transient effect in Go cells. Also from the data obtained it seems that the Retinal action on cytoplasmic proteins and proteins released to the culture medium is transient and opposite. These variations could be related to the different capacities of retinoid-treated cells to grow in a semi-solid agar medium. Cells treated for 60 days give rise to macrocolonies of loosely packed cells while smaller colonies are obtained with cells treated for 4 months (data not shown), probably reflecting the concentration or the quality of the proteins released to the culture medium. Finally, although there are many literature examples on the action of retinoids in differentiation

and carcinogenesis (for a review, see ref. 18), their mechanism of action could be tissue and cell specific. Results obtained in our laboratory (19) show that 13-cis Retinal induces the proliferation of MCF-7 populations which have a lower grade of differentiation. From the above results it is evident that long-term cellular and biochemical studies should be done before considering retinoids as "cancer preventive agents".

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