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RETINOIC ACID BLOCKADE OF IMIDAZOLE-INDUCED TYROSINASE EXPRESSION IN B16 MELANOMA CULTURES: SIMILAR EFFECTS OF THE ACTIVE RETINOID AND TRIIODOTHYRONINE

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SUMMARY: The effect of retinoic acid on the induction of tyrosinase (EC 1:14.18.1) by imidazole was determined in cultured B16/C3 melanoma cells. Retinoic acid could block the induction of enzyme activity within 3 hours of addition to the culture medium at a physiological concentration (10nM). The blockade was similar to that of 3,3',5-L-triiodothyronine (T_3) already reported. mRNA hybridizable to a tyrosinase DNA probe was induced by imidazole while retinoic acid and T_3 blocked that increase. These observations suggest that retinoic acid can mimic the action of T_3 in B16 melanoma cells in culture.

Mouse tyrosinase (EC 1.14.18.1), the rate limiting, copper containing enzyme involved in melanogenesis, exists as multiple isoforms (1). Several cDNA clones have been isolated from murine melanoma libraries (2,3), the most abundant transcript apparently encodes a functional enzyme (3), a 533 amino acid peptide which contains an 18 amino acid residue leader sequence (4). Alternative splicing is involved in the generation of multiple transcripts (3).

The murine melanoma cell line, B16/C3, is a useful model for examining the regulation of melanogenesis in neoplastic tissue. Our laboratories have reported recently that 3,3',5-L-triiodothyronine (T_3) but not thyroxine can inhibit the activity of tyrosinase in B16 cells in culture (5,6). In addition, T_3 can block the induction of that enzyme by imidazole (5,6), an action which the active iodothyronine shares with estradiol (7) and testosterone (8). Thus the chemical induction of tyrosinase in this cell line is under multi-hormonal control (9).

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The recent identification of a family of protooncogenes which encode receptors for T_3 (10,11) as well as several steroid hormones (12) has allowed a careful examination of the primary gene and receptor protein structures. The c-erb A gene apparently encodes a nuclear T_3 receptor. A retinoic acid receptor has also been identified as belonging to this receptor family (13,14) and its DNA binding domain apparently shares a 62% primary sequence homology with the T_3 receptor. The demonstration that the retinoic acid receptor binds to a thyroid hormone response element and activates gene expression of primers fused to that element (15) suggests the possible overlap in responsive networks for thyroid hormone and retinoic acid.

Retinoic acid has been shown to retard the proliferation of human melanoma cells in culture (16) and to inhibit melanogenesis in murine melanoma cultures (17). This communication describes the results of studies which examine the influence of retinoic acid on imidazole-induced tyrosinase expression in B16 cell cultures. The retinoid blocked induction of the enzyme by imidazole. Thus retinoic acid influences melanogenesis in these cultured cells in a similar manner to T_3.

MATERIALS

Chemicals

T_3 and retinoic acid were purchased from Sigma (St. Louis, MO). [^{3}H]-tyrosine (specific act.52.5 Ci/mmol) and [^{32}p]dCTP were obtained from New England Nuclear (Boston, MA) and ICN (Irvine, CA), respectively. Imidazole was from Fisher Scientific (Pittsburgh, PA).

Cell Culture

B16/C3 mouse melanoma cells were stored in liquid nitrogen, thawed and plated in 25 cm flasks (Corning, Medifield, MA) covered with minimal essential medium supplemented with donor calf serum (10%) in a 37° C incubator. Cells were removed for passage in calcium-free, magnesium-free phosphate buffered saline (PBS) containing 0.05% EDTA, pH 7.4. Experimental flasks were inoculated with 1.5 x 10^5 late exponential cells and were allowed to attach for 6 h before any experimental manipulations were performed. Fresh medium containing imidazole was added and the cultures were allowed to proliferate, usually for 18 h. T_3 and/or retinoic acid were added at the times denoted in the legends to the figures. Control cultures received equivalent volumes of diluent (95% ethanol). The final imidazole and T_3 concentrations were 10 mM and 50 nM, respectively.

Tyrosinase Assay

Tyrosinase activity in whole cell sonicates was determined as described previously (18). Briefly, monolayers were rinsed

thoroughly in PBS containing EDTA (0.05%) and suspended in sodium phosphate buffer (80 mM, pH 6.8), and frozen at -20° C until assayed. Samples were thawed and sonicated at 4° C with two 15s bursts with a microtip probe (Heat Systems model W-220F, Plainview,NY). The assay mixture contained 0.4 umol L-tyrosine, 0.04 umol, 3,4-dihydroxy-L-phenylalanine in sodium phosphate buffer (26 umol, pH 6.8) and 2.5uCi [^{3}H]tyrosine in addition to 0.2 ml cell sonicate suspension (total reaction volume 0.4 ml). Reactions were carried out at 37° C for 1 h in a shaking water bath and terminated with the addition of ice cold trichloroacetic acid to a final concentration of 10% (v/v). Samples were allowed to precipitate to completion at 4° C followed by the addition of activated charcoal suspension (Norit A, Fisher, 100 mg/ml final concentration) to extract unreacted tyrosine. They were then subjected to centrifugation and an aliquot of the resultant supernatant was assayed for radioactive content by liquid scintillation spectroscopy. Protein concentrations were assayed by the method of Lowry et al. (19).

The abundance of mRNA hybridizable to a 22 base-pair DNA probe which corresponds to the first exon of the tyrosinase structural gene was determined as follows: Proliferating cultures were treated with the appropriate additives for 2 h in fresh medium. Culture monolayers were washed and solubilized in a solution containing guanidine thiocyanate (4 M) and RNA was extracted (20). Equivalent amounts of RNA were immobilized on nitrocellulose filters by the method of Gillespie and Bresser (21) and quick blot hybridizations were carried out with the DNA probe labeled with [^{32}p]dCTP under the stringency conditions recommended (21).

RESULTS AND DISCUSSION

Imidazole induces tyrosinase in B16 melanoma cells in culture (18). The compound increased the activity of that enzyme by approximately 100% when present for 18 h, as figure 1 demonstrates. The addition of T_3 (50 nM) blocks the induction of tyrosinase by imidazole so that the enzyme activity in cultures receiving both compounds is similar to that in control cultures (5). That concentration of T_3 is known to inhibit maximally the imidazole induction (5). Retinoic acid (10 nM) could also block block the imidazole induction (Fig. 1). When retinoic acid and T_3 were added together, there was no further decrease in enzyme activity.

The induction of enzyme activity associated with imidazole treatment is accompanied by an increase in the accumulation of mRNA hybridizable to the tyrosinase DNA probe (Fig. 2). As that figure demonstrates, both T_3 and retinoic acid lower the mRNA content in imidazole-induced cultures to levels below that seen in controls.

Retinoic acid, like T_3, can block the induction of tyrosinase by imidazole. These effects on enzyme activity are

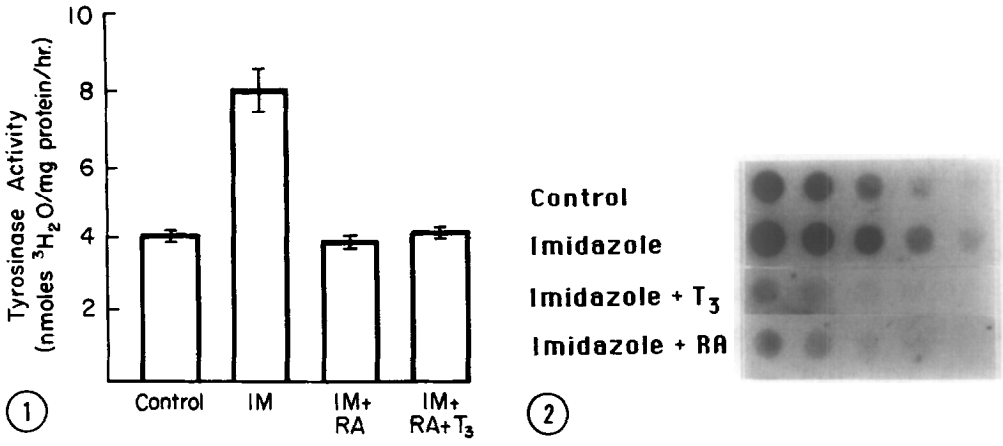

Figure 1. Effects of retinoic acid (RA) and T_3 on the induction of tyrosinase by imidazole (IM). Proliferating cultures were incubated in the presence of imidazole (10mM) for 12 hours followed by the addition of retinoic acid (10nM), T_3 (50nM) or the combination of the two for 3 more hours. Cultures were harvested and tyrosinase activity determined as described in Methods. Each column represents the mean ± SE of triplicate cultures from one representative experiment.

Figure 2. Effects of imidazole, T_3 (50nM) or retinoic acid (RA) (1nM) on the abundance of RNA hybridizable to a tyrosinase probe. 4ug of total cellular RNA was immobilized to yield the left-hand blots followed by serial 2-fold dilutions. All blots were from a single filter processed in a single hybridization. Cultures were treated for 2 h with the respective supplements.

correlated with the abundance of tyrosinase mRNA. While the mechanism(s) involved in the iodothyronine as well as the retinoid effects on tyrosinase are not fully elucidated, they likely involve a modulation of gene expression. In the case of T_3, we have demonstrated previously that inhibitors of de novo protein and RNA synthesis block the hormone effects (5). Retinoic acid may have influenced the transcriptional rate or may have altered the turnover of the tyrosinase transcript. Thyroid hormone regulation of gene expression appears to involve both mechanisms (22-24). Our previous results suggest that T_3 does not influence the stability of the translatable mRNA encoding tyrosinase (5).

Other laboratories have reported retinoid action at the genomic level. Retinoic acid has been reported capable of inducing differentiation in vitro (25). A recent report by Kim and Wolf demonstrated a 2-4 fold increase in fibronectin mRNA levels in the liver of vitamin A-depleted rats and in cultured rats hepatocytes, an action which the authors attributed to an increased rate of transcription (26).

The finding that thyroid hormone and retinoic acid have similar actions on a specific cellular process such as melanogenesis suggests a possible relatedness between these structurally diverse molecules, consistent with the recent findings of Umesono and colleagues (15). Their demonstration of a shared gene response element implies a potential for overlap in gene response networks.

There exists reason a priori to suspect that thyroid hormone and retinoids are functionally related. Kunde, in 1926, reported that hypothyroid rabbits developed hypovitaminosis A (27). Retinoid metabolism is influenced by the thyroidal state (28) and retinoids are capable of influencing thyroidal economy (29) and can block the action of iodothyronines at the red cell membrane (30). The observations reported here suggest the possibility the T_3 and retinoic acid modulate the expression of the same gene and are consistent with a shared nuclear site of action.

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