Stimulation of the receptor for melanocyte-stimulating hormone by retinoic acid

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Treatment of Cloudman S91 melanoma cells with retinoic acid (RA) inhibits MSH-induced tyrosinase activity and melanin formation [(1990) J. Invest. Dermatol. 94, 461–464]. We report here, however, that in spite of inhibiting MSH-induced pigmentation, RA treatment caused a marked increase in MSH binding capacity for both cell surface and internal MSH binding sites. The stimulation was dose- and time-dependent and reversible, with half-maximal effects seen at 2 μM RA. Stimulation of MSH binding was seen as early as 3 h after exposure of cells to RA. Cell surface and internal binding activity increased in concert. Scatchard analyses indicated that increased MSH binding resulted from a 3–4-fold increase in the number of sites with no significant difference in their affinity for MSH. It appears that in suppressing MSH-induced melanogenesis, RA elicited a compensatory up-regulation of the MSH receptor system.

Melanoma; Melanotropin receptor; Retinoic acid

1. INTRODUCTION

Retinoic acid (RA) has a wide ringe of biological effects on normal and transformed cells in culture and in vivo. In particular, teratocarcinoma, neuroblastoma and melanoma cell lines stop proliferation and in some cases exhibit altered phenotypic expression in response to RA treatment (for review see [1]). Melanin synthesis has been used as an indicator of melanoma cell differentiation, and there have been several reports showing that retinoic acid can act as either a stimulator or suppressor of basal melanization in cultured melanoma cells [2–5]. We studied the effects of retinoic acid on inducible melanization, either hormonally with melanocyte stimulating hormone (MSH), or pharmacologically with cholera toxin, isobutylmethylxanthine (IBMX), and found that in all cases retinoic acid is a potent inhibitor of the induction process [6]. This was true not only for retinoic acid, but for a number structurally unrelated compounds which are known in other systems to induce differentiation (dimethylsulfoxide, hexamethylene bisacetamide, sodium butyrate). We were surprised to find, however, that even though these agents each inhibited melanocyte-stimulating hormone-induced melanogenesis, they did not inhibit MSH binding capacity of the melanoma cells, and in fact markedly stimulated MSH binding [7]. It appeared that in suppressing induced melanogenesis, the agents elicited a compensatory up-regulation of the MSH receptor system. This report details the nature of the increased binding capacity of Cloudman melanoma cells for MSH following exposure to retinoic acid.

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2. MATERIALS AND METHODS

2.1 *Cell line and culture conditions*

The Cloudman S91 murine melanoma cell line 'PS1-HGPRT-1' was used in all studies [8]. Cells were cultured in Corning tissue culture flasks at 37°C, 90% humidity and 5% CO_2 in Ham's F10 medium supplemented with horse serum (10%), penicillin (100 IU/ml) and streptomycin (100 mg/ml). Cells were passaged twice weekly after removal from the substratum with Joklik's medium containing 1 mM EDTA.

2.2 *Treatment of cells with retinoic acid*

Retinoic acid was obtained from Sigma Chemicals (St. Louis, MO), dissolved at 10 mM in DMSO, and stored at −20°C kept in foil-wrapped containers protected from light. Cells were plated for 12–24 h in plain culture medium prior to any experimental additions. At the end of treatment, cells were removed from substratum by incubation with Joklik's buffered saline containing EDTA (1 mM), counted with a Coulter counter and pelleted by centrifugation.

2.3 *Binding of ^{125}I-β-MSH to intact cells*

Iodination of β-MSH and isolation of the mono-iodinated peptide (retaining full biological activity as assayed by its ability to stimulate tyrosinase) by reverse phase liquid chromatography were carried out in this laboratory by a modification [9] of the method of Lambert and Lerner [10]. The binding of ^{125}-I-β-MSH to cells was assayed as previously described [11]. Non-specific binding ranged from 5 to 10% of the total binding, and was subtracted to generate the displayed values.

2.4 *Internal binding sites for ^{125}I-β-MSH*

Cells were removed from flasks, chilled to 4°C, pelleted by centrifugation (700 × *g*, 10 min), resuspended in hypotonic buffer (10

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mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCL_2$, pH 7.8) at 4°C, and lysed by passages through needles as previously described [11]. The lysate was then centrifuged at 156000 × *g* for 20 min. The pellet fraction was then resuspended in MSH binding buffer (140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 0.1% glucose) incubated with ^{125}I-β-MSH (1 nM) in the presence or absence of a 5000-fold excess of non-radioactive β-MSH in a shaking water bath (10°C) for 90–120 min, layered onto a stepwise sucrose gradient (8–80%), and centrifuged at 156000 × *g* for 60 min. Fractions were collected and refractive indices determined as described [11].

2.5 *Statistical analysis*

Statistical analyses of the data were done using the STATVIEW program (Abacus). *P* values were calculated from one-tail paired *t*-test using the same program.

3. RESULTS

3.1 *Dose-response*

The relationship between MSH binding and the concentration of RA administered was determined in Cloudman S91 melanoma cells pretreated with various concentrations of RA for 20 h (Fig. 1). The MSH binding was stimulated by RA, with a significant ($P < 0.05$) increase (160 ± 16% of control) detected at RA concentrations greater than 10^{-6} M. Maximal stimulation, (223 ± 23% of control, $P < 0.05$) was observed at 4×10^{-6} M RA.

3.2 *Time course and reversibility*

Stimulation of MSH binding by RA as a function of time was determined. Fig. 2 shows hormone binding by cells exposed to 4×10^{-6} M RA up to 42 h. A significant increase in MSH binding was detected in treated cells after 3 h (116 ± 8% of control, $P < 0.01$). Maximal binding was observed after 18–24 h (195 ± 29% of control, $P < 0.0001$). This stimulation was reversible.

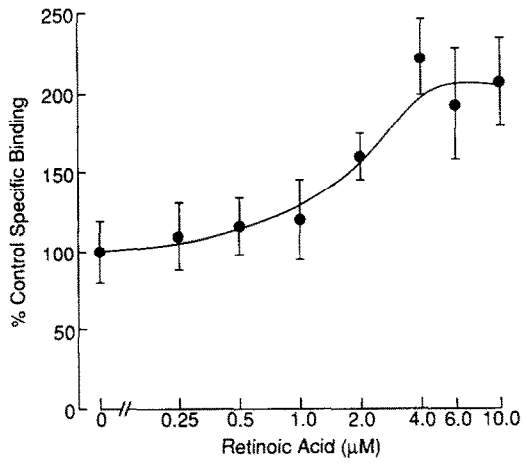

Fig. 1. Dose-dependent stimulation of MSH receptor activity by RA. Cloudman S91 mouse melanoma cells were incubated with indicated doses of RA for 20 h. Specific counts bound were determined as described in section 2. Results are expressed as percentage of the control specific binding. Each point represents mean ± SD of three determinations from a representative experiment. The experiment was repeated twice with similar results.

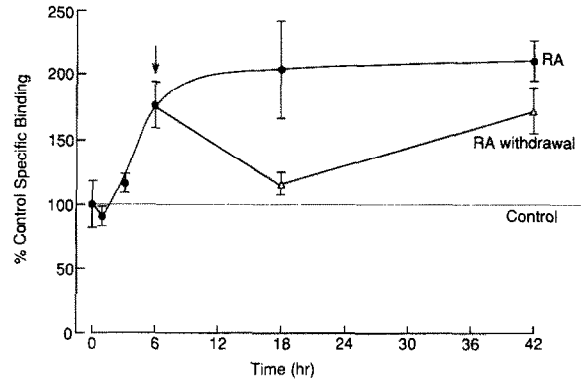

Fig. 2. Time-dependent effect of RA on MSH receptor activity and its reversibility. Cloudman S91 mouse melanoma cells were incubated with 4 μM RA for the indicated time periods, after which the cells were taken up by Joklik's buffered saline containing 1 mM EDTA for MSH receptor binding assay as described in section 2. The arrow indicates the point of retinoic acid withdrawal. Values represent the mean ± SD of 3 separate experiments done in triplicate.

The increased binding detected in cells treated with 4×10^{-6} M RA for 6 h returned to normal within 24 h after removal of drug. 36–48 h after removal of RA, MSH binding activity consistently rose to levels even higher than those obtained by initial treatment with the drug.

3.3 *Binding competition and Scatchard analysis*

To further characterize the effect of RA treatment on MSH binding, the cells were preincubated for 20 h with either RA (4×10^{-6} M) or DMSO (0.02%) as control, and the binding of ^{125}I-β-MSH was measured in the presence of various concentrations of unlabelled hormone (Fig. 3). In untreated cells, the half-maximal inhibition of binding was achieved by the inclusion of 7 nM unlabelled hormone, whereas in RA-treated cells 30 nM unlabeled hormone was required to effect the same inhibition.

Scatchard analyses of the binding data showed a curvilinear plot which could be explained either by a two-site model or by negative cooperativity. Using a two-site model and computerized linear regression analysis, we found that untreated cells expressed an average of 6317 ± 2025 (mean ± SD) high affinity MSH binding sites per cell, whereas RA-treated cells expressed 28200 ± 6700 high affinity sites per cell (Fig. 3, inset). The number of 'low affinity' sites was 65900 ± 8600 for control cells and 263900 ± 75800 for RA-treated cells. Differences in equilibrium dissociation constants between control and RA-treated cells were insignificant, approximate K_d values being 1 nM and 15 nM for high and low affinity sites, respectively, suggesting that the mechanism by which RA increased MSH binding capacity involved increasing the number of MSH receptors.

3.4 *Internal binding sites*

We have previously described the presence of internal

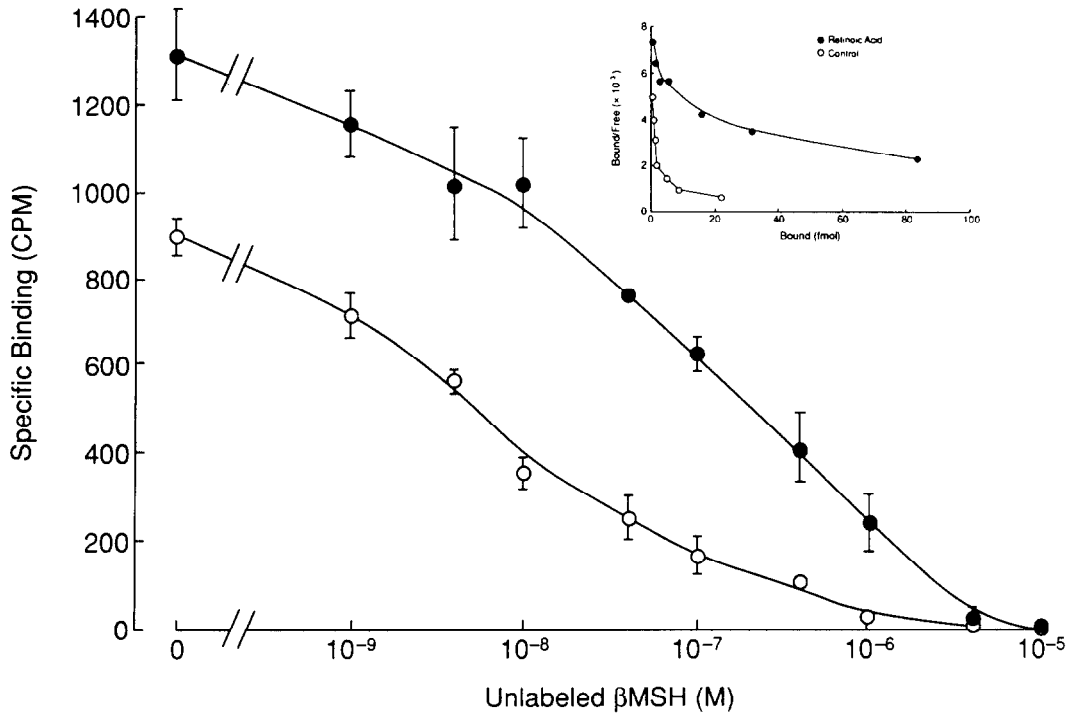

Fig. 3. Competition-inhibition curves of ^{125}I-β-MSH binding with unlabelled β-MSH in Cloudman S91 cells treated (●) or untreated (○) with RA (4 μM) for 20 h. Cells (2.5 × 10^5) were incubated in the presence of 1 nM ^{125}I-β-MSH plus the indicated concentrations (abscissa) of unlabelled β-MSH. The values are the mean ± SD of three determinations at each concentration of unlabelled β-MSH. The experiments were repeated twice with similar results. Scatchard analyses of the data are shown in the inset.

binding sites for MSH in Cloudman melanoma cells, and demonstrated that these sites correlated with cellular responsiveness to the hormone [11]. We therefore determined whether such sites were also affected by RA treatment of the cells. When the dense particulate fraction from treated cells was analyzed for the presence of MSH binding activity, a large increase (245 ± 22% control, $P < 0.01$) which paralleled that of the surface receptors was noted (Fig. 4). Because of the technical difficulties involved in the manipulation of these fractions, Scatchard analysis could not be performed to determine whether this increase was a function of altered receptor affinity, receptor number, or both.

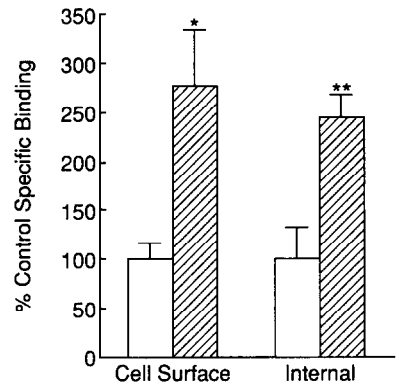

Fig. 4. Effect of RA on internal MSH binding sites from Cloudman S91 cells. Cells were treated with ▨ or without RA □ (4 μM) for 36 h. After treatment, cell surface MSH binding sites and internal MSH binding sites were assayed. Results are expressed as percentage of control specific binding. The experiment was repeated three times with similar results. Data points represent the mean ± SD of triplicate determinations from a representative experiment. Asterisks indicate values significantly different from control at *$P < 0.001$ and **$P < 0.01$.

4. DISCUSSION

Retinoic acid increased the binding of MSH to its cell surface receptor in a time- and dose-dependent manner. The optimum concentration and time were found to be 4 μM and 24 h, respectively. The effects were reversible within 24 h after removal of the drug. Scatchard analysis of the competitive inhibition of ^{125}I-β-MSH binding by non-radioactive hormone indicated that RA treatment increased the receptor number of both the high and low affinity sites per cell by 3–4-fold. The receptor number for untreated cells (approximately 6000) was similar to the numbers reported by others for Cloudman melanoma cells [10,12].

The biochemical basis for receptor up-regulation by RA could be due to an increased rate of synthesis, a decreased rate of receptor degradation, a blocking of internalization, or a stimulation of externalization of receptor. Two pieces of evidence mitigate against a role for alterations in receptor internalization or externalization as the cause of cell surface up-regulation. First,

both internal and surface receptor activity were increased by RA in concert. Second, although retinoic acid blocks the effects of MSH on melanogenesis, it does not interfere with MSH-induced changes in cellular morphology and growth, suggesting that at least the initial steps in the signal transduction pathway are not disturbed by RA [6,7].

The regulation of MSH receptor levels has important biologic implications. MSH causes an increase in cellular levels of cAMP [13], stimulation of tyrosinase activity and melanin content [14], as well as changes in growth and morphology of Cloudman S91 melanoma cells [15]. Yet, despite its stimulatory effects on MSH binding, RA inhibits MSH-induced tyrosinase activity and melanin production [6], but not MSH-induced changes in the rate of cell proliferation or morphology [7]. Therefore, RA causes only a partial modulation of the cellular responses to MSH suggesting that the effects of MSH on pigmentation, growth, and morphology may be regulated through separate pathways. The independent and possibly alternative pathways for the regulation of these two cellular events have been suggested by others [16,17].

For certain cell lines, MSH binding is strongly dependent on cell cycle and cell density [18]. Our binding experiments were performed on confluent monolayer cultures, and the effects of RA on the cell cycle as they relate to MSH binding remain to be studied more extensively.

RA alone has been shown to inhibit proliferation and clonogenicity of numerous melanoma cell lines in vitro, and has been used parenterally and topically [19] to inhibit melanoma growth in experimental animals. On the other hand, the MSH receptor has been suggested as a target for antimelanoma chemotherapy via the use of hormone toxin/conjugates (e.g. daunomycin) [20,22] or by the use of hormone/antibody conjugates to enhance cytotoxic lymphocyte killing of melanoma cells [22]. The current study raises the intriguing possibility of combining both RA and MSH-toxin or antibody conjugates in one treatment regimen. It may be possible that RA would not only inhibit tumor growth but might greatly enhance the effects of the hormone conjugates by up-regulating cellular receptors for the hormone.

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