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Agouti Protein Inhibits Growth of B16 Melanoma Cells *in Vitro* by Acting through Melanocortin Receptors

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Agouti protein is known to antagonize cAMP formation, tyrosinase activation and melanogenesis in mouse B16-F1 melanoma cells induced by α-melanocyte-stimulating hormone (α-MSH). We now demonstrate that although agouti binds to the melanocortin receptor MC1-R with an almost identical affinity to that of α-MSH, it does not antagonize the inhibitory action of α-MSH on the growth of B16-F1 cells. Instead it has a similar antiproliferative action with a half-maximal effective concentration of 13 nM. In G4F cells lacking MC1-R, agouti is without effect. Agouti was also found to induce MC1-R down-regulation with identical kinetics and magnitude as α-MSH. Thus, the different effects of agouti on B16-F1 cells proceed via interaction with MC1-R but are not exclusively antagonistic.

Melanogenesis in mammalian melanocytes leads to the formation of either eumelanin or pheomelanin, resulting in a brownish-black or yellowish-red coat color, respectively (1). The switch between these two pathways is controlled by the products of two classical coat-color controlling gene loci, *extension* and *agouti* (2). *Extension* encodes for the melanocortin receptor MC1-R, which is coupled to G proteins. MC1-R binds α-melanocyte-stimulating hormone (α-MSH) and is expressed on pigment cells (3–6). The 131-amino acid *agouti* gene product acts in a paracrine manner to shift melanogenesis towards pheomelanin (7). In addition, when agouti is overexpressed ectopically in yellow mutants, it induces obesity, insulin resistance and increased susceptibility to neoplastic lesions (8, 9). Agouti has been shown to compete with α-MSH in binding to MC1-R and to antagonize MSH-induced cAMP formation (10, 11). However, some recent data (12) support the hypothesis (13) that agouti can also act via an additional mechanism that differs from its ligand antagonism of MC1-R. In the present study, we demonstrate a novel function of agouti, namely the inhibition of melanoma cell proliferation. Moreover, we present evidence that the expression of MC1-R is a prerequisite for this effect.

MATERIALS AND METHODS

Materials. α-MSH was kindly supplied by Ciba-Geigy AG, Basel, Switzerland. [Nle^4, D-Phe^7]α-MSH was purchased from Bachem AG, Bubendorf, Switzerland.

Production of murine agouti. Briefly, the 614-bp XbaI/PstI fragment of the murine agouti cDNA was subcloned into the baculovirus vector pAcMP3 (Pharmingen, San Diego, CA). Virus incorporating this vector was produced by standard methods (14). Isolation and purification of murine agouti and assessment of its activity were performed as described (10,11). Generally, the preparation contained 90% pure agouti.

Cell culture. B16-F1 (15) and G4F (16) mouse melanoma cells were grown in modified Eagle's medium (MEM) with Earle's salts (Gibco BRL Life Technologies, Paisley, Scotland) in the presence of 10% heat-inactivated FCS, 2 mM L-glutamine, 1% MEM non-essential amino acids (100x; Gibco), 1.5% MEM vitamin solution (100x; Gibco), penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were kept in a humidified atmosphere of 5% CO_2 at 37°C and subcultured twice weekly after detaching with 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free PBS (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.44 g $Na_2HPO_42H_2O$ per liter).

Cell proliferation assay. Proliferation was measured with a dye colorimetric method (17). B16-F1 cells were seeded at a density of 200 cells per well in 96-well culture dishes. The cells were allowed to adhere for 24 h before adding the reagents

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to a final volume of 250 μl. After 72 h of incubation (5% CO_2, 37°C), the culture medium was removed by a flick of the wrist and the cells were fixed with 100 μl of 10% (wt/vol) formaldehyde containing 0.9 g/100 ml NaCl for at least 30 min. After removing the fixative, the cells were stained with 100 μl of filtered 1% (wt/vol) methylene blue in 0.01 M borate buffer (pH 8.5) for 30 min at ambient temperature. The wells were then washed with 4 × 200 μl borate buffer and the dye was eluted with 100 μl of 1:1 (vol/vol) ethanol and 0.1 M HCl overnight at 4°C. Absorbance at 650 nm was measured with a microplate reader (Molecular Devices, Menlo Park, CA).

Melanin assay. Melanin production by B16-F1 cells was measured in 96-well microplate culture dishes as described (18).

Radioiodination of [Nle^4, D-Phe^7]α-MSH. Radioiodination of [Nle^4, D-Phe^7]α-MSH with Na^{125}I (NEN, DuPont) was performed using lactoperoxidase and glucose oxidase on a solid phase support (Enzymobeads, BioRad, Richmond, CA) as described previously (19). The iodination product was purified on a 1-ml syringe packed with 0.4 cm^3 Spherisorb ODS/10 μm reversed-phase silica by elution with a stepwise increasing gradient of methanol in 1% TFA. Mono-iodinated [Nle^4, D-Phe^7]α-MSH eluted at 49.5-50% methanol. The radioligand was used within 14 days after iodination. Before each experiment, an aliquot of the tracer was additionally purified by reversed-phase HPLC on a Spherisorb ODS/5 μm column using 0.1% TFA (solvent A) and 0.1% TFA/70% acetonitrile/30% water (solvent B) with a linear 16-min gradient from 50 to 75% solvent B, flow rate 1.2 ml/min. The peak fraction eluting at 65 to 66% solvent B was collected and lyophilized.

MC1 receptor binding and down-regulation. Binding studies with dispersed B16-F1 cells were performed as previously described (20). Briefly, cells were detached with EDTA and counted in a Coulter counter. Binding with [^{125}I]-[Nle^4, D-Phe^7]α-MSH was performed in MEM with 25 mM HEPES (Gibco), 0.2% BSA, and 0.3 mM 1,10-phenanthroline (Merck, Darmstadt, FRG). After 3 h of incubation at 15°C, bound radioligand was separated from free radioactivity by centrifugation through silicon oil and measured in a gamma counter (Canberra Packard). MC1-R down-regulation was assessed on cells grown in 96-well culture strip plates (Costar, Cambridge, MA). B16-F1 cells were seeded at a density of 1 × 10^4 cells per well and cultured for 48 h. α-MSH, agouti, or vehicle were added to a final volume of 200 μl culture medium and the plates were incubated at 37°C. After 0, 30, 90, and 180 min, the plates were placed on ice and carefully washed twice with ice-cold acidic buffer (40 mM sodium acetate, pH 4.5, containing 0.9% NaCl and 10% FCS) and once with binding medium. Equilibrium binding was then performed with [^{125}I][Nle^4, D-Phe^7]α-MSH in 100 μl binding medium at room temperature for 2 h. Non-specific binding was assessed in the presence of 1 μM α-MSH. The plates were then washed twice with ice-cold binding medium and single wells containing the cell monolayers were broken off and counted in a gamma counter.

Data analysis. Data are presented as means with standard deviations. Binding data were analyzed using the LIGAND programs (21). Dose-response curves were nonlinearly fitted according to a 4-parametric logistic function.

RESULTS AND DISCUSSION

Wild-type murine agouti protein dose-dependently inhibited growth of B16-F1 mouse melanoma cells (Fig. 1), similar to α-MSH (22). The maximal effect of 40% growth reduction was achieved in the concentration range of 100-300 nM, and the half-maximal effective concentration (EC_{50}) of agouti was 13 nM (Fig. 1). This is 20-fold higher than that of α-MSH (EC_{50} 0.65 nM; data not shown) which raises the question whether the antiproliferative effect of agouti is mediated by

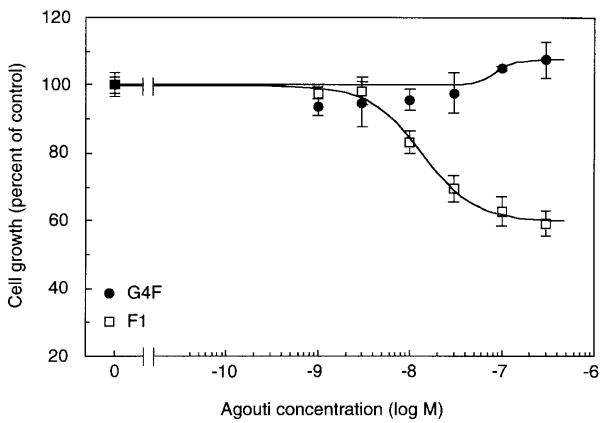

FIG. 1. Dose-dependent growth effects of agouti on B16-F1 and G4F cells. Cells were incubated for 72 h with increasing concentrations of wild-type mouse agouti protein. Proliferation was assessed colorimetrically after methylene blue staining. The data represent the mean and SD of triplicate determinations.

TABLE 1
Binding Affinities of α-MSH and Agouti to MC1-R

	α-MSH	Agouti
K_D (nM)	2.3 ± 0.2	3.7 ± 0.9

Dissociation constants (K_D) were determined by Scatchard analysis of equilibrium binding experiments with suspended cells at 15°C using [^{125}I][Nle^4,D-Phe^7]α-MSH as radioligand. A K_D of 0.42 nmol/l was obtained for the radioligand. The data represent mean values ± SD from three experiments.

MC1-R. The studies were therefore extended to G4F cells, a B16 melanoma cell variant that lacks MC1-R both at the protein and the mRNA level (16). Agouti did not induce growth inhibition in these cells. Instead, a minor growth stimulation occurred following exposure of G4F cells to 100 and 300 nM agouti (Fig. 1). Whether this slight stimulatory effect of agouti is the result of an interaction with a receptor that is different from MC1-R but recognizes agouti specifically, is not yet clear. In B16-F1 cells, however, the antiproliferative activity of agouti appears to result from binding to MC1-R.

We then determined whether the binding characteristics of agouti to MC1-R in B16-F1 cells differed from those of α-MSH. By using Scatchard analysis, agouti and α-MSH were found to bind to a single class of high-affinity binding sites and to display very similar dissociation constants (Table 1) indicating that agouti is a more potent ligand in B16-F1 cells than other melanocortin peptides such as ACTH (20).

To further investigate the significance of agouti and α-MSH interactions at the level of MC1-R with respect to melanogenesis, we determined their effects on melanin production using the *in situ* melanin assay (Table 2). Although agouti was able to antagonize MSH-induced melanin formation, a 100 to 1000-fold molar excess of agouti was required for complete inhibition of the α-MSH effect. In G4F cells, neither agouti nor α-MSH elicited significant effects on melanin production, when given alone or in combination (data not shown). Thus, although agouti binds to MC1-R with an affinity equal to that of α-MSH, inhibition of α-MSH-induced melanogenesis only becomes apparent in a concentration range where agouti has been shown to reduce cell growth.

Another aspect of interest is the question whether agouti influences MC1-R regulation, since α-MSH has been shown to induce down-regulation of MC1-R in B16-F1 and other mouse melanoma cells but up-regulation in some human melanoma cells (23). Figure 2 demonstrates that

TABLE 2
Melanin Production by B16-F1 Cells in Presence of α-MSH or α-MSH and Agouti

Additions	Melanin (A_{405})
none	0.291 ± 0.004
α-MSH (0.1 nM) alone	0.979 ± 0.011
α-MSH (0.1 nM) + agouti (10 nM)	0.602 ± 0.023
α-MSH (0.1 nM) + agouti (100 nM)	0.285 ± 0.003
α-MSH (1 nM) alone	1.117 ± 0.024
α-MSH (1 nM) + agouti (10 nM)	1.100 ± 0.012
α-MSH (1 nM) + agouti (100 nM)	0.288 ± 0.001

B16-F1 cells were incubated with α-MSH or α-MSH and agouti as indicated. After 72 h, melanin production was assessed by measuring the absorbance at 405 nm.

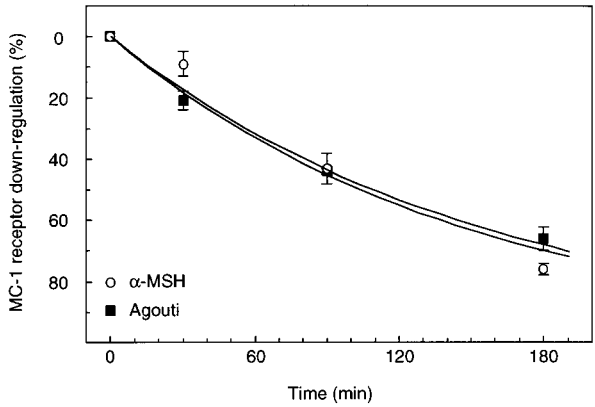

FIG. 2. Time-course of MC1-R down-regulation induced by α-MSH and agouti. B16-F1 cells were incubated at 37°C with 3 nM α-MSH or 3 nM agouti for the indicated time periods. Bound ligands were then released by a short acid wash and the number of surface receptors assessed by radioligand binding at 20°C.

agouti induced MC1-R down-regulation and that the characteristics of this down-regulation were virtually identical to those observed for the α-MSH-induced MC1-R down-regulation. The concentration range at which agouti was effective was the same as that of α-MSH (3 nM), which means that agouti affects receptor regulation at a 100-fold lower concentration than that required for inducing growth inhibition or for blocking α-MSH-induced melanogenesis. These findings support the idea that the function of agouti is not solely to inhibit the binding of α-MSH to its receptor since, in general, antagonists do not induce down-regulation of G protein-coupled receptors (with the exception of serotonin receptors) (24).

In conclusion, we demonstrate for the first time an antiproliferative action of agouti protein on B16 cells *in vitro* and MC1-R down-regulation and confirm high-affinity binding of agouti to MC1-R as well as inhibition of α-MSH-induced melanogenesis. Whether these effects of agouti all proceed via a single signalling cascade or via different cascades, needs further investigation. One explanation for the antiproliferative action of agouti (and possibly the inhibition of melanogenesis) could be that fact that agouti down-regulates MC1-R, resulting in lower levels of intracellular second messengers, such as cAMP, which are required for normal cell growth and function. Alternatively, agouti may bind to MC1-R in such a way that the agouti-receptor complex does not only activate the G_s/adenylate cyclase signalling pathway but may couple to additional effector molecules. Such types of coupling have been described for other G protein-coupled receptors (25). Thus, although the different effects of agouti on B16-F1 cells proceed via interaction with MC1-R, they are not exclusively antagonistic.

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