Studies on the Cloudman Melanoma Cell Line as a Model for the Action of MSH

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INTRODUCTION

In the early part of this century it was discovered that experimental removal of pituitary glands from tadpoles resulted in rapid lightening of their integument, and that addition of pituitary extracts to the water in which these animals were living reversed the effect, causing integumental darkening [1,2]. Subsequent studies revealed that the active ingredients of the pituitary extracts belonged to a class of closely related peptides today known interchangeably as melanotropins, melanocyte stimulating hormones, or simply MSH. Melanotropins are synthesized as part of a larger precursor molecule, pro-opiomelanocortin [3]. Three forms have been described, α, β, and γ, ranging in size from 12 to 18 amino acids. Each of the three peptides has sequence homology to adrenocorticotropic hormone (ACTH) and to β-lipotropin (β-LPH). MSH, as these peptides will henceforth be referred to, is active throughout the vertebrate kingdom in stimulating the pigmentation system [4,5], although the mechanisms of action differ between the lower vertebrates and mammals. In addition to stimulation of the pigmentation system, there are a number of other functions that might be assigned to MSH; for example, in regulation of synaptic transmission [6] and in aldosterone biosynthesis in adrenal glomerulosa cells [7]. Whether or not a cell is able to respond to MSH is likely to depend on the ability to express high affinity receptors for MSH and to exhibit biochemical pathways that are regulated by the formation of the MSH-receptor complexes [8]. In 1973, Wong and Pawelek first demonstrated that Cloudman S91 mouse melanoma cells growing in culture respond to MSH with increases in pigmentation, as well as alterations in morphology and rates of proliferation [9]. Since that initial observation, there have been a number of studies with these cells on various aspects of the mechanisms of action of MSH. Evidence has been
obtained to support the following conclusions: (a) MSH binds to specific, high-affinity receptors on the cell surface [10,11]; (b) formation of the hormone-receptor complex is followed by a stimulation of the adenylate cyclase system and a net increase in intracellular levels of cyclic AMP (cAMP) [12,13]; (c) the hormone-receptor complex is internalized and apparently migrates to premelanosomes, the intracellular sites of melanin synthesis [8]; (d) the increased levels of cAMP result in increased tyrosinase (EC 1.14.18.8) activity and melanin deposition as well as changes in morphology and rates of proliferation [9]; and (e) these processes are mediated, at least in part, by cAMP-dependent protein kinases [14–16].

The purpose of this article is to review the evidence in support of the above conclusions focusing on the research done at Yale, as well as to present some recent, and as yet unpublished, findings which extend these ongoing studies.

ISOLATION OF GENETIC VARIANTS OF THE CLOUDMAN MELANOMA LINE

In 1973, it was first shown that MSH can reduce the growth rate of Cloudman melanoma cells in culture [9]. This observation opened the way for isolating mutant cell lines which were resistant to these suppressive effects of MSH on proliferation. Subsequent studies resulted in the establishment of techniques for isolating pure lines of cells that did not respond to MSH and showed no changes in either pigment formation, morphology, or rates of proliferation [17,18]. Analyses of the defects in these mutants allowed us to conclude that there are common biochemical pathways for the control of different cellular functions by MSH. For example, cells selected for resistance to MSH in growth frequently also showed no increase in pigmentation in response to MSH, even though no selective pressure had been applied for pigmentation. Since the appearance of a particular mutant trait is a rare event (one in 10^6) the chances of having a second trait occur as an independent mutation and be isolated accidentally with no selective pressure would be extremely rare, statistically 10^{-6} \times 10^{-6}, or one in 10^{12}. It is therefore virtually impossible to isolate double mutants without selective pressure. The more likely conclusion is that a single mutational event affected both proliferation and pigmentation, and reciprocally in "normal" melanoma cells a common biochemical step controls both functions. One common biochemical pathway controlling responses to MSH is the cyclic cAMP system.

THE ROLE OF CYCLIC AMP IN THE RESPONSES TO MSH

The responses to MSH are apparently mediated through increases in intracellular levels of cAMP. MSH causes a rapid and dramatic increase in cAMP levels. The action of MSH is mimicked by analogues of cAMP such as dibutyryl cAMP or 8-bromo cAMP, as well as agents that raise intracellular levels of cAMP, such as cholera toxin, prostaglandin E_1, theophylline, or isobutylmethylxanthine. Some mutant cell lines that do not respond to MSH with changes in growth or pigmentation also do not show increases in intracellular levels of cAMP [18].

CYCLIC AMP-DEPENDENT PROTEIN KINASE

Kuo and Greengard's observations on the widespread occurrence of cAMP-dependent protein kinases throughout the phyla suggested that these enzymes are the mediators of the many and varied responses to cAMP [19]. This situation appears to be true, at least in part, for MSH and Cloudman melanoma cells. Two lines of evidence
are as follows. First, it was shown that the activity of tyrosinase, a rate-limiting enzyme in melanin biosynthesis, is increased in intact cells by MSH or by analogues of cAMP [9,18]. In 1977, it was reported that this increased tyrosinase activity could be achieved in a test tube using extracts of melanoma cells, cyclic cAMP, ATP, magnesium, and a partially purified cAMP-dependent protein kinase prepared from the same cells [14]. Second, it was shown that a cAMP-dependent protein kinase from Cloudman cells is involved in the regulation of proliferation by MSH [15]. The involvement of cAMP-dependent protein kinases in these functions implies that protein phosphorylation/dephosphorylation reactions are part of the biochemical pathway influenced by MSH [16].

ROLE OF PHOSPHOPROTEINS

A number of observations led us to conclude that phosphoproteins are important in the regulation of proliferation and pigmentation. Indirect evidence, discussed above, comes from the findings that the action of MSH is mediated through cAMP-dependent protein kinase activity. Through direct analyses, we have observed as many as eight proteins whose phosphorylation is regulated by MSH [16]. This phenomenon is highly complex in that there are several subsets of regulatory steps involved. Some proteins show decreased phosphorylation in lysates of cells exposed to MSH; others show increased phosphorylation. One group of proteins of approximately 47,000 daltons shows increased phosphorylation when cAMP is included in the reaction mix, but only in cells not exposed to MSH. None of the other MSH-regulated proteins is affected by cAMP in the in vitro reaction mix. Phosphorylation of three proteins is regulated "qualitatively" by MSH in that exposure to the hormone seems to result in a switch between phosphorylation of tyrosine residues and phosphorylation of serine and threonine residues. This finding could be extremely important, because phosphorylation of tyrosine residues has recently been linked to the control of growth. The possibility is raised that the "phosphotyrosine proteins" are those involved in growth regulation, while the others are involved in regulation of melanogenesis.

NEW REGULATORS OF THE MELANOGENSES PATHWAY

Melanin is formed spontaneously from dopaquino in a test tube. This fact led to a generally accepted conclusion that the only catalyzed steps in melanogenesis are the conversion of tyrosine to dopa and of dopa to dopaquino by tyrosinase. However, recent studies, beginning with those of Logan and Weatherhead on Siberian hamsters, have indicated that there are additional regulatory points governing the Mason-Raper pathway [20–26]. Using amelanotic Cloudman cells which lacked tyrosinase activity, we showed that dopachrome conversion factor accelerates the conversion of dopachrome into 5, 6-dihydroxyindole-2-carboxylic acid, and that indole blocking factor inhibits the conversion of 5,6-dihydroxyindole (5,6-DHI) into melanochrome [21–23]. Also, we showed that melanotic cells possess an activity that accelerates the conversion of 5,6-DHI into melanochrome and that this activity can at least in part be accounted for by tyrosinase itself [24]. Therefore, this unusual enzyme can catalyze three reactions in the synthesis of melanin [24]. In addition we have observed in Cloudman cells and in skins from neonatal mice an activity, distinct from that of tyrosinase, which also accelerates conversion of 5,6-DHI into melanochrome [25]. The nature of this activity is as yet unknown.
Barber and King have confirmed and extended our observations of dopachrome conversion factor and indole blocking factor activities [26]. They have measured these activities in a variety of different sources, including human hair bulbs. Their studies have led them to conclude that the activities can be accounted for by a single enzyme. We have as yet been unable to separate dopachrome conversion factor and indole blocking factor activities from each other, consistent with the "single enzyme" proposal of Barber and King. However, it should be pointed out that until the activities are purified to homogeneity, no definitive statement regarding this situation is possible [27].

Taken together, the results from three separate laboratories support the notion that there are regulatory steps in the Mason-Raper pathway following the formation of dopaquinone. To our knowledge, these regulated steps have been observed exclusively in eumelanogenic systems, and it remains to be seen whether they also are involved in pheomelanogenesis. It is our feeling that many additional regulatory events in these pathways exist. In this regard, we found that in some, but not all experiments, melanin formed by extracts of skins from brown mice was brown, whereas melanin formed by extracts of skins from sombre mice and from black mice was always black [25]. This was surprising since differences in color between brown and black mice were considered to be due to size and shapes of melanosomes rather than color of the melanin [28]. Since we observed differences in color between the melanins synthesized in the absence of melanosomes, perhaps there are determinants in skin extracts which regulate formation of brown or black melanin.

CYTOTOXICITY OF MELANIN PRECURSORS

From a variety of clinical and experimental observations, Lerner proposed several years ago that, during the process of melanization, a pigment cell produces substances which are potentially autotoxic [29]. Since that proposal, several laboratories have confirmed that the precursors of melanin biosynthesis are highly toxic to melanoma cells, probably because of their ability to generate free radicals as they co-polymerize. We showed that tyrosine and dopa are toxic to melanoma cells in culture and that the toxicity is increased significantly if the cells are pre-exposed to MSH [13,23]. We also showed that other intermediates, namely dopachrome and 5,6-DHI, are even more toxic than tyrosine and dopa [23,30]. However, cells that synthesize melanin possess mechanisms for protecting themselves from the cytotoxic intermediates, and our evidence indicates that, in Cloudman melanoma cells, the protective mechanism is related to the relative activities of tyrosinase, dopachrome conversion factor, and indole blocking factor.

SYNTHESIS OF ¹²⁵I-LABELED β-MSH FOR ANALYSES OF MSH RECEPTORS

Freychet, Roth, and Neville reported the synthesis of ¹²⁵I-labeled insulin in 1971 [31]. This achievement opened the way for studies on receptors for insulin and led to the development of techniques for iodination and isolation of many biologically active peptide hormones. Varga et al. [10] first reported the synthesis of ¹²⁵I-labeled β-MSH and the use of this ligand to investigate MSH receptors in cultured melanoma cells. Lambert, Lerner, and Varga later described an improved synthesis and purification of ¹²⁵I-β-MSH which resulted in the isolation of a radioactive ligand that was mono-iodinated at a single tyrosine residue and that retained full biological activity when
compared to non-radioactive $\beta$-MSH [11,32,33]. Their methods employed the use of IodoGen (1, 3, 4, 6-tetrachloro-3, 6-diphenylglycoluril, Pierce Chemicals, Inc.) as a catalyst during the iodination reaction, and isolation of the $^{125}$I-$\beta$-MSH by reversed phase high-performance liquid chromatography. Recently, the number of studies on receptors for MSH has increased markedly, as has a concomitant need for a supply of $^{125}$I-MSH. Improvements on the original techniques have now led to methodology wherein millicurie amounts of $^{125}$I-MSH can be isolated and stored for at least six weeks [34]. The continued advancement of this and related technology [35] has been of major importance to progress in the studies on the structure and function of receptors for MSH.

CELL-CYCLE SPECIFIC EXPRESSION OF RECEPTORS FOR MSH

In 1974 it was reported that at least some of the responses to MSH (e.g., increased cyclic AMP production and tyrosinase activity) occur in the G2 phase of the cell cycle [36] and that the apparent reason for this cell cycle restriction is that receptors for MSH are most active in the G2 phase [10]. However, in the earlier work, the mechanism(s) for increased receptor activity were not studied; e.g., were the receptors increased in number or in affinity? We recently examined these phenomena in detail. We found that by three separate methods of obtaining populations of cells in the G2 phase of their cycle—centrifugal elutriation, or synchronization with either colchicine or thymidine—there was increased binding of MSH by cells in the G2- and possibly late S-phases of their cycle. However, cultures of cells passing through their cycle in synchrony were quite different from non-synchronized (random) cultures. Both synchronized and random cultures expressed receptors for MSH in the G2- and possibly late S-phase of their cycle, but synchronized cultures bound 15–20 times more MSH per cell than random cultures. This increased binding of MSH by synchronized cells was accompanied by an increase in tyrosinase activity, providing direct proof of a relationship between cellular binding of MSH and cellular responsiveness to MSH. Analyses by Scatchard and Hill methods indicated that receptors from synchronized cells exhibited positive cooperativity, while receptors from random populations of cells exhibited no cooperativity [37].

Understanding the nature of these differences in receptor populations should be of fundamental value in understanding the mechanism of action of the MSH-receptor complex in regulating proliferation and pigmentation.

DOPA PHOSPHATES: A NEW CLASS OF COMPOUNDS WHICH REGULATE PIGMENT BIOSYNTHESIS AND CELLULAR RESPONSIVENESS TO MSH

One problem in many of the studies on the regulation of pigmentation is that the key metabolite, namely L-dopa, is relatively insoluble (maximum solubility is approximately 5 mM in aqueous solution at neutral pH), and is converted readily to melanin in the presence of $O_2$ above pH 7. Thus many studies with cultured pigment cells or with melanomas in animals are difficult to carry out or to interpret when L-dopa is a component of the experiment. With this problem in mind, procedures were designed for attaching phosphate groups to the oxygens on the 3- and/or 4-positions of the phenylalanine ring [38]. The rationale was that such compounds would be stable to oxidation and far more soluble than dopa itself. It was reasoned that, if melanocytes were able to take up these compounds, the cellular phosphatases might catalytically
hydrolyze the phosphate groups and in the process produce free L-dopa. Results of our studies with these compounds are summarized as follows: Dopa phosphates are highly soluble compounds which are stable over a wide range of pH values and are not hydrolyzed by boiling in concentrated acid. Synthetic yields of greater than 90 percent can be obtained using dopa as starting material. Exposure to alkaline phosphatase results in hydrolysis of the phosphate moieties and production of dopa. Dopa phosphates do not inhibit dopa oxidase (tyrosinase) activity. Dopa oxidase does not catalyze the conversion of dopa phosphates into melanin unless the dopa phosphates are first treated with phosphatase. Dopa phosphates, when compared to L-dopa, are stable in the presence of O2 and are not oxidized by serum proteins. In the presence of cultured melanoma cells, dopa phosphates are readily converted into melanin, indicating that the cells are able to produce dopa from dopa phosphates. At high concentrations, dopa phosphates are cytotoxic toward melanoma cells in culture. The cytotoxicity is enhanced at least threefold by pretreatment of cells with melanotropin (MSH), and prevented by phenylthiourea, an inhibitor of dopa oxidase activity. These results, combined with studies on the uptake of radioactive forms of dopa phosphates (32P and 14C) indicate that phosphorylated isomers of dopa are efficiently taken up by Cloudman melanoma cells and are readily converted by the cells into a melanin precursor, presumably L-dopa [39].

The above findings with dopa phosphates were obtained when the compounds were used at relatively high concentrations (10^-3M). A surprising finding emerged during investigations into possible effects of dopa phosphates on the activity of receptors for MSH [40]. Using 125I-MSH as a tracer, we found that preincubation of Cloudman melanoma cells with very low concentrations of dopa phosphates (10^-6-10^-5M) resulted in a three- to fourfold stimulation of MSH receptor activity. In addition, the stimulation of receptor activity was followed by a marked increase in MSH-mediated stimulation of tyrosinase activity and melanin deposition. Scatchard analyses indicated that the stimulation of receptor activity occurred through an increase in the affinity of the receptors for MSH rather than through an increase in the number of receptors. Apparently, at low concentrations dopa phosphates act either directly or indirectly as cofactors for the MSH receptors. It is important to note that, at the low concentrations, dopa phosphates did not increase tyrosinase activity or melanin content; rather they increased the cellular responsiveness to MSH [40].

The effects of dopa phosphates on melanoma cells in culture were consistent with findings in similar in vivo studies on SKH2 mice [41]. At high concentrations (0.1 percent) pigmentation was suppressed, while at low concentrations (0.01 percent) pigmentation was enhanced. It is possible that the reduction in skin pigment with high concentrations of dopa phosphates was due to the cytotoxic effects of melanin intermediates described above, while the stimulation of pigmentation with low concentrations of dopa phosphates was due to a stimulation of receptors for MSH.

CONCLUSIONS

Hormonal regulation of cellular proliferation and the expression of differentiated functions in a number of cell types is a topic of considerable attention. As with most biological studies, the increased number of observations has underlined the diversity of the topics and raised more questions than originally imagined. The studies on Cloudman melanoma cells and their regulation by MSH presented here represent a good example of this situation. Although we have made several observations on the
regulation of proliferation and pigmentation, the findings have indicated that the
regulation of these functions is highly complex and still poorly understood. Nonethe-
less, the fact that these cells grow in culture and respond to MSH, the ability to
manipulate them genetically, the development of radioactive MSH as a tracer, and the
development of new techniques such as production of monoclonal antibodies and the
ability to clone genes should all be of assistance in the future progress of work on this
system.

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