MOLECULAR BIOLOGY OF PIGMENT CELLS

Molecular Controls in Mammalian Pigmentation

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Genetic regulation in bacteria, fungi, yeast, and higher plants has been extensively studied, two well-known systems being the lactose (1) and arabinose (2) operons in Escherichia coli. The understanding of the mechanisms by which these systems function has depended heavily upon (a) the ability to obtain cells bearing mutations, thus allowing for genetic analyses of the various complementation groups involved, and (b) the ability to obtain sufficient biological material to perform biochemical analyses of the complementation groups. Progress in the understanding of genetic regulation in animals has advanced more slowly and is proportional to the increased difficulties in performing genetic and biochemical analyses.

Cultured mammalian melanoma cells provide excellent material for studying the regulation of phenotypic expression in eucaryotic cells because:

1. The oxidation of tyrosine to melanin is well understood. A single enzyme, tyrosinase, is thought to control this pathway. A simple and rapid assay for tyrosinase activity in cells growing in situ and in cell homogenates is available (3).

2. Melanin in the cells can be detected visually and thus provides an easy assay for the expression of a differentiated function.

3. Isogenous pigmented cell lines can be established by clonal techniques, and from these lines amelanotic variants can be isolated. At least some of these variants should have genetic lesions in the melanin pathway. A genetic analysis of this differentiated function should be possible.

4. A melanoma cell line has been found which responds dramatically to melanocyte stimulating hormone (MSH), showing large increases in tyrosinase activity and melanin content following exposure to the hormone. Use of this cell line should facilitate studies of the biochemical controls of pigment formation (4–5).

5. Kilogram quantities of melanoma cells can be obtained by growing the cells as tumors in animals.

This report is a summary of recent biochemical and genetic studies in our laboratory on mammalian melanoma cells grown in clonal and monolayer culture and as tumors in animals. The paper is divided into the following sections:
I. Control of phenotypic expression of cultured melanoma cells by MSH.
II. Establishment of isogenic cell lines from a mouse melanoma. Isolation and analysis of amelanotic variants.
III. Studies of the effect of tyrosine on the growth of melanoma cells in culture and in animals.

**I. CONTROL OF PHENOTYPIC EXPRESSION OF CULTURED MELANOMA CELLS BY MSH**

Studies of the mechanism through which MSH acts in mammals have been restricted to intact animals or to melanomas grown in animals (6-10, 41). We recently described a mouse melanoma cell line cultivated in monolayer which, when exposed to MSH, showed large increases in tyrosinase activity and melanin content, as well as changes in growth characteristics and cellular morphology (4-5). We also found that adenosine 3',5'-monophosphate (cyclic AMP) or its analogue N6,O2-dibutyryl cyclic AMP can substitute for MSH. This finding supports previous evidence that MSH probably acts through cyclic AMP in mammals (6, 8, 11).

Cloudman S91 NCTC 3960 (CCL 53) mouse melanoma cells were obtained from the American Type Culture Collection Cell Repository (12). If the cultures are inoculated at a relatively low cell density ($5-10 \times 10^4$ cells/250 ml Falcon tissue culture flask) and subcultured weekly, they have low tyrosinase activity and are only lightly pigmented. If, however, MSH is added to the culture medium, a dramatic increase in pigmentation is seen within 2-4 days (Fig. 1).

The cells were examined with the electron microscope (Figs. 2a and b). In the absence of MSH most cells contained numerous premelanosomes, but in the pres-

*Fig. 1. Effects of MSH on pigment formation by cultured melanoma cells. The culture medium was 10 ml Ham's nutrient mixture F10, supplemented with 10% horse serum, 2% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1.2 mg/ml sodium bicarbonate. 10⁶ mouse melanoma cells were inoculated into each of 4 culture flasks. MSH ($10^{-7} M$, purified by Drs. Upton, Lerner, and Lande) was added to two of the flasks. After 4 days the cells were harvested, pooled, lysed in hypotonic buffer, and photographed. MSH-treated cells are on the left.*
Fig. 2. Electron microscope studies of the effects of MSH on cultured melanoma cells. Cells were grown in the absence (a) or presence (b) of MSH as in Fig. 1. After 4 days, they were harvested, fixed in 3% glutaraldehyde followed by 1% osmium tetroxide, and embedded in epoxy resin (63,000×). We thank Ms. Elizabeth Godawska and Dr. Gisela Moellmann for these studies.
ence of MSH most cells contained fully melanized (stage IV) melanosomes. Thus it appears that the synthesis of melanosomes is not a rate-limiting step in the response to MSH, although it is possible that pre-melanosomes lack specific factors that regulate melanization which are made available following exposure to MSH.

One obvious rate-limiting factor in melanization could be the availability of tyrosinase. This enzyme was measured in both the presence and absence of MSH. It was found that, preceding the increase in melanization, there was a large increase in tyrosinase activity (4-5). A kinetic study of tyrosinase activity, however, reveals that there is a "silent period" of approximately 6-9 hr after exposure to MSH (Fig. 3). Only after this period was the increase in activity detected. If the hormone was removed after 24 hr, the activity continued to increase for an additional 24 hr. It then gradually fell to the level found in untreated cells. We do not yet know whether the MSH-mediated increase in tyrosinase activity represents new synthesis of tyrosinase or activation of pre-existing molecules. Studies are currently underway on this important question (13).

We investigated the roles of RNA and protein synthesis in the response to MSH (4-5). Actinomycin D and cycloheximide were used to inhibit RNA and protein synthesis, respectively. The drugs inhibited the synthesis of RNA and proteins to more than 90% but had no cytotoxic effects during the time periods that they were studied. MSH was added for 24 hr and then withdrawn. Tyrosinase activity was measured during hours 0-24 and 24-48. The inhibitors were added during the first or second time intervals. Cycloheximide inhibited the response to MSH during both time intervals, indicating that protein synthesis is continuously necessary.

Fig. 3. Kinetics of induction of tyrosinase activity following exposure of melanoma cells to MSH. Cells were inoculated and treated with MSH as in Fig. 1. Cellular tyrosinase activity was assayed in situ. L-tyrosine-3,5-3H (New England Nuclear) was added to the culture medium at 1.0 μCi/ml with a final specific activity in the medium of 96 μCi/μmole. At appropriate times, 0.5 ml medium was removed, and 3H2O was measured by the charcoal absorption method of Pomerantz (3). The counts represent incremental 3H2O formation during each time interval. (X---X, △---△) MSH-treated cells; (●---●, ○---○) control cells.
Fig. 4. Effects of MSH on intracellular cyclic AMP concentration in cultured melanoma cells. Cells were cultured as in Fig. 1. At appropriate time intervals, they were harvested, and cyclic AMP was measured by the method of Brown et al. (15). We thank Gary Pretzold in the laboratory of Dr. Paul Greengard for the cyclic AMP assays.

Fig. 5. Cyclic AMP binding activity in extracts of cultured melanoma cells. Cells were cultured as in Fig. 1. They were harvested and then lysed in 24 mM Tris, pH 7.5, 0.5% Triton X-100, and 4 mM theophylline. A 0.1-ml reaction mix contained $10^4$ lysed cells in the above buffer and 2 $\mu$Ci $^3$H-cyclic AMP (New England Nuclear, 22 Ci/mmole). The mix was kept on ice for 60 min, and then free cyclic AMP was separated from bound on a Sephadex G-25 column equilibrated with 25 mM Tris, pH 7.5.

Actinomycin D inhibited the response if present during the first 24 hr or "silent period," but had no effect on tyrosinase activity during the second 24 hr. Apparently, genetic expression is a prerequisite for increased tyrosinase activity.
TABLE 1

| Nonradioactive nucleotide | ×[^3]H-cAMP (pmoles/ml) | Counts bound | % of control |
|---------------------------|-------------------------|--------------|--------------|
| cAMP                      | 0                       | 6985         | 100          |
|                           | 1 ×                     | 5130         | 74           |
|                           | 10 ×                    | 1693         | 24           |
|                           | 100 ×                   | 672          | background   |
|                           | 1000 ×                  | 420          | background   |
| 5’AMP                     | 10 ×                    | 7888         | 113          |
|                           | 100 ×                   | 8241         | 118          |
| cUMP                      | 10 ×                    | 7109         | 103          |
|                           | 100 ×                   | 7497         | 107          |
| cCMP                      | 10 ×                    | 7264         | 104          |
|                           | 100 ×                   | 7978         | 114          |
| cGMP                      | 10 ×                    | 7346         | 105          |
|                           | 100 ×                   | 7837         | 112          |

**a. In presence of non radioactive nucleotides**

**b. Following proteolysis**

| Treatment           | Counts bound | % of control |
|---------------------|--------------|--------------|
| None                | 33850        | 100          |
|                     | 36100        |              |
| Pronase + trypsin   | 3350         | <10          |
|                     | 3150         |              |
| Pancreatic RNase    | 34750        | 100          |
|                     | 33900        |              |
| Pancreatic DNase    | 37500        | >100         |
|                     | 39870        |              |

Within minutes after exposure to MSH, intracellular levels of cyclic AMP rose dramatically and then returned to near basal levels in a few hours (Fig. 4). An extensive kinetic study of intracellular cyclic AMP levels during the 48-hr exposure to MSH is currently underway.

Evidence from many laboratories indicates that cyclic AMP acts intracellularly by activating protein kinases which in turn elicit responses by the phosphorylation of specific proteins (e.g., 15). Thus, in the melanoma cell, proteins that bind to cyclic AMP may be intracellular mediators in the response to MSH. Gold et al. (8) have recently reported cyclic AMP-dependent protein kinase activity in melanomas. In extracts of cultured melanoma cells we have found proteins that bind[^3]H-cAMP (Fig. 5). The binding was specific in that several related nonradioactive compounds did not complete with[^3]H-cAMP (Table 1a). Binding activity was greatly reduced when the extract was preincubated with proteolytic enzymes (Table 1b). Isolation and analyses of the cyclic AMP-binding proteins should be useful for understanding the mechanism of action of MSH.

Several questions, summarized schematically in Fig. 6, have been raised here concerning molecular controls in mammalian melanogenesis. Many of these topics should be amenable to experimental analysis.

II. ESTABLISHMENT OF ISOGONOUS CELL LINES FROM A MOUSE MELANOMA. ISOLATION AND ANALYSIS OF AMELANOTIC VARIANTS

In recent years techniques have been developed that permit genetic analyses of regulatory systems in cultured animal cells. Puck and his coworkers (16–17) de-
vised procedures for selectively isolating mutant animal cells in culture and performed complementation analyses of nutritionally deficient cells using the cell–cell hybridization techniques developed by Ephrussi (18), Harris (19), Littlefield (20), and others (for a review, see 21). In many laboratories, cell culture and somatic cell hybridization are being used to investigate the regulation of phenotypic expression in differentiated cells. Experiments have been designed so that a cell expressing a readily assayable trait is fused with a cell not expressing that trait (see 22). These experiments are too numerous to summarize here. The results, however, have generally been:

(a) The parental trait is extinguished in the progeny (e.g., 23).
(b) The trait is not extinguished and persists in the progeny (e.g., 24).
(c) The progeny express new traits that are not seen in the parents (e.g., 25).
(d) The parental trait is expressed in the progeny only if the parent with the trait has at least twice its normal chromosome complement prior to fusion (e.g., 26–27).

Interpretations of the results are as yet difficult. These types of experiments, however, promise progress in the studies of regulation of phenotypic expression.

We are approaching the problems somewhat differently. We are in the process of gathering clones of stable amelanotic melanoma cells which are the progeny of melanotic parents. We expect that at least some of these amelanotic variants have acquired genetic lesions in their pigmentation pathway and are mutants. By hybridizing mutants with one another, we will be able to order the genetic defects into the complementation groups. Once the number of complementation groups is established, and hence at least a minimum number for the genes governing pigmentation, we hope to study them by genetic and biochemical analyses much as the lactose and arabinose mutants are being studied in Escherichia coli. We are using simple and rapid cloning techniques (28) which allow the isolation of single amelanotic clones from large populations of melanotic clones.

Establishment of isogenous cell lines. The mouse melanoma of Cloudman, S91, Clone M-3 (CCL-53.1) was obtained from the American Type Culture Collection.
Repository (12) and initially maintained in monolayer culture. To establish isogenous pigmented lines, cells from M-3 were cloned in soft agar (28). M-3 was chosen for genetic studies for the following reasons:

1. The cells clone well in soft agar, forming large, highly pigmented colonies within 2–3 weeks (Fig. 7). Several other melanoma cell lines, including the S91-3690 line used in the MSH studies, were found unsuitable for cloning in soft agar, either because they grew poorly or were only lightly pigmented.

2. Subclones of M-3 possess a phenotype of stable pigmentation and are highly pigmented even in the absence of MSH.

3. M-3 cells fuse readily with Sendai virus (29).

Several pigmented clones were independently isolated from M-3 cells and, in turn, subcloned at least 10 times before being screened for amelanotic variants. After 10 subclonings the lines were considered to be isogenous. Several of the lines were morphologically distinguishable from one another in monolayer culture.

**Isolation of amelanotic variants.** One isogenous subline of M-3, designated PS 4-wt (wild type), was selected for screening for amelanotic variants. At this writing, we have screened several million clones of PS 4-wt and isolated 5 stable amelanotic variants. These have remained amelanotic through at least 5 subclonings. Approximately 100 additional white clones were isolated, but were unstable and eventually became pigmented. These clones were discarded.

Figure 8 shows amelanotic clone PS 4-3 compared to the melanotic parental clone PS 4-wt. The frequency of occurrence of stable amelanotic variants is approximately $1 \times 10^{-6}$. We are treating the cells with various mutagenic agents with the hope that we can increase this frequency.

Amelanotic clones PS 4-3 and PS 4-4 are now growing vigorously in monolayer culture. As yet, the only detectable differences from the wild type are in tyrosinase

![Fig. 7. Melanoma cells growing clonally in soft agar. An isogenous cell line, designated P-S 4-wt was derived from the Cloudman melanoma clone M-3. In this photograph, the cells have been cultured for 3 weeks. Each clone represents approximately 7,000 cells.](image)
activity (approximately 5% of wild type) and melanin content (negligible). PS 4-3 was examined with the electron microscope. The cells contained numerous pre-melanosomes; however, melanized melanosomes were absent (see Fig. 2 of Dr. Gisela Moellmann’s paper, this volume).

Genetic complementation studies are currently underway with the amelanotic variants. These studies are in the preliminary stages and will be reported later.

III. THE EFFECT OF TYROSINE ON THE GROWTH OF MELANOMA CELLS IN VIVO AND IN VITRO

From a variety of clinical and experimental observations, it can be stated that, in the process of melanization, a pigment cell produces substances toxic for that cell (30). Normal pigment cells may have a mechanism that is protective, so that the toxic effects from melanin production do not interfere with the viability of the cells (31).

There are numerous reports that pigmented melanocytes are selectively killed by phenolic compounds, such as hydroquinone (e.g., 32–33), and by mercaptoethylamines, such as cysteamine (34). The effects of hydroquinone were known as early as 1936, when it was reported that this chemical when fed to black cats induced the formation of gray hair (35). For further discussions of depigmenting agents see Refs. (30, 36). Generally, the result with the depigmenting agents has been that in vivo, pigmented melanocytes are selectively killed. V. Riley studied the effects of the compounds para- and ortho-phenylenediamine on malignant melanoma in mice and hamsters (37). He was encouraged to find that these compounds caused tumor regression and apparent remission for the animals. Unfortunately, when used in concentrations necessary for tumor regression in humans, these agents had strong toxic side effects (V. Riley, personal communication).

We initiated studies aimed at finding agents which showed selective toxicity toward melanotic melanoma cells, but which might be tolerable when administered clinically to humans.

![Fig. 8. Isolation of amelanotic variants. An amelanotic clone designated P-S 4-3 is pictured on the left. On the right is a melanotic clone from the parental line P-S 4 (wt).](image-url)
Cells in culture. We tested cysteamine, hydroquinone, dopa, tyrosine, and N-acetyltirosine for selective toxicity toward cultured melanotic melanoma cells. Five separate cell lines were tested for sensitivity to these compounds: Vero green monkey kidney cells (nonpigmented); an amelanotic mouse melanoma of Cloudman, S-91 NCTC 3959 (CCL-52); 2 melanotic mouse melanomas of Cloudman, S-91 NCTC 3690 (CCL-53) and S-91 clone M-3 (CCL-53.1); and the melanotic hamster melanoma of Greene. Cysteamine, hydroquinone, and dopa were highly toxic to all the cell lines and, in our hands, had no selective toxicity toward melanotic cells. On the other hand, tyrosine and N-acetyltirosine were toxic to the melanotic but had little effect on the amelanotic cells.

Dose-response studies revealed that tyrosine was more efficient than N-acetyltirosine in that the optimal tyrosine concentration to produce toxicity (about 5 mM) was tenfold lower than the optimal N-acetyltirosine concentration. Therefore, subsequent experiments dealt exclusively with the effects of tyrosine on melanoma cells. For example:

Cells were inoculated into Falcon 100-mm petri dishes at $2 \times 10^6$ cells per dish, and were grown as in Fig. 1. After 2 days growth, some of the cultures were supplemented with 1 mg/ml tyrosine. Daily measurements were made of the number of cells and the amount of DNA per culture dish. Visual observations of the cells were made with a phase microscope. Usually, within 4 days, all pigmented cells exposed to tyrosine died, while nonpigmented cells showed a retarded growth rate but were otherwise unaffected (Fig. 9). We found variability in these experiments, but that was the exception rather than the rule.

Tumors in animals. We tested the effects of tyrosine on melanomas in hamsters and mice. Two examples:
1. DBA-2J mice were injected with the melanotic melanoma of Cloudman (Clone M-3), and Syrian Golden hamsters were injected with the melanotic hamster melanoma of Greene. The animals were inoculated subcutaneously with \(2 \times 10^6\) cells. After 2 days, some animals were placed on a diet of chow containing 33\% (wt/wt) tyrosine. After 3–4 weeks, the animals were sacrificed, and the tumors removed and weighed. Animals kept on the high-tyrosine diets had tumors strikingly smaller than those from animals on control diets. There was a 2–5-fold reduction in tumor size for animals on diets high in tyrosine \( (p = 0.05)\).

2. Animals were inoculated with tumor cells, placed on the above diets, and some animals on each diet were given daily injections of melanocyte stimulating hormone (MSH). Results indicated that MSH increased tumor size in animals on control diets but decreased even further tumor size in animals on high-tyrosine diets.

A more detailed description of these experiments is being prepared (39).

**DISCUSSION AND CONCLUSION**

Several experiments have been presented, all dealing with the control of pigmentation in mammalian melanoma cells. A number of questions may now be amenable to experimental analysis.

1. Are new molecules of tyrosinase synthesized, are pre-existing molecules activated, or are both of these mechanisms involved?
2. What specific forms of genetic expression are involved?
3. What are the molecular intermediates?
4. Does MSH act solely through cyclic AMP, or are other pathways operative?
5. Why are many established melanoma cell lines pigmented in the absence of MSH?
6. How many genetic complementation groups govern pigmentation?
7. What are the roles of the complementation groups?

We are interested primarily in regulatory gene mutations, but we expect that some mutations will be in structural genes such as those coding for the tyrosinase enzymes or structural proteins of the melanosome. One way to enrich for regulatory gene mutants is by the use of temperature sensitivity. For example, if we were to isolate a temperature-sensitive mutant clone which is amelanotic at 38°C and exhibits no tyrosinase activity, but is melanotic at 33°C with normal tyrosinase activity, we would then isolate tyrosinase from the cells. If the tyrosinase itself were not temperature sensitive, the findings could indicate that the mutation resided in a gene regulating tyrosinase synthesis.

It would be of great value to establish cell-free systems for the transcription and translation of tyrosinase genes. For example:

(a) A system might be established from cells that have not been exposed to MSH. Regulation of the expression of tyrosinase genes might then be accomplished by adding fractions from cells that have been exposed to the hormone, including cyclic AMP binding proteins.

(b) A system might be established from cells that are amelanotic mutants. Gene expression might be regulated by adding fractions from wild-type cells.

Establishment of such systems could thus aid the identification of molecular pathways involved in the control of melanogenesis.
In the selective toxicity of tyrosine toward melanotic melanoma cells:
1. What is the molecular basis of this toxicity?
2. Can tyrosine or dopa be used chemotherapeutically in the treatment of human melanoma? Will MSH potentiate the chemotherapy?

The mechanism(s) by which the various depigmenting agents are cytotoxic to melanocytes is unknown. A possible one, however, comes from the work of Hochstein and Cohen (31), who provide evidence that polyphenolic intermediates in the formation of melanin from tyrosine are potentially cytotoxic. Perhaps related to the findings of Hochstein and Cohen is the "self-destruct" hypothesis of Lerner (30), which proposes that melanocytes produce autotoxic factors (possibly melanin precursors or their byproducts) which are normally sequestered but which in disease states such as vitiligo cause destruction of the cells. Such a self-destruct mechanism could explain the occasional spontaneous regression of malignant melanoma (40).

For a variety of reasons, cultured melanoma cells are appealing models for studies of phenotypic expression in mammals.

SUMMARY

Experimental approaches are described for studying the control of phenotypic expression in mammalian melanoma cells. These approaches include (a) investigation of the mechanism of action of melanocyte stimulating hormone (MSH) in the control of melanization, (b) isolation and analysis of amelanotic cell lines from melanotic parents, and (c) studies of selective toxicity of tyrosine toward melanotic melanoma cells. A cultured melanoma cell line is described which responds dramatically to MSH with increases in intracellular cyclic AMP, tyrosinase activity, and melanin deposition. Methods are presented for establishing isogenous melanotic cell lines and for the isolation of amelanotic variants from these lines. Some preliminary analyses of the variants are given. Results are presented which show that high levels of tyrosine are selectively toxic toward melanotic melanoma cells in culture and in animals, and that, in animals, MSH may potentiate the toxicity.

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