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Isolation and Characterization of a Variant of B16-Mouse Melanoma Resistant to MSH Growth Inhibition

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A variant of B-16 F_1 mouse melanoma was selected for its ability to survive and replicate in the presence of melanocyte-stimulating hormone (MSH). Although the variant (MR-4) was completely resistant to growth inhibition by MSH, cyclic AMP was still able to block cell replication. Tyrosinase activity in MR-4 cells was considerably lower than in B-16 F_1 cells. MSH induced a twofold to threefold increase in tyrosinase activity in both cell types, but the absolute activity in MR-4 remained significantly less than in the parental cells. MR-4 cells were also found to have a markedly depressed cyclic AMP-dependent protein kinase activity relative to B-16 F_1 cells. The protein kinase from both cell types was stimulated by cyclic AMP, but the level of MR-4 kinase activity at maximal cyclic AMP concentrations remained considerably lower than B-16 F_1 kinase activity under the same conditions. In both cell types adenylate cyclase activity was markedly stimulated by MSH. When equal numbers of viable F_1 and MR-4 cells were injected subcutaneously into C57/B1 mice, the MR-4 cells formed tumors earlier and killed the host sooner than the parental F_1 cells. We conclude that the biochemical alteration which allows MR-4 cells to replicate in the presence of MSH is a low level of tyrosinase activity, which in turn may be the result of low cyclic AMP-dependent protein kinase activity.

Key words: **tumorigenicity, cyclic AMP-dependent protein kinase, tyrosinase MSH-growth-resistant variant, mouse melanoma**

Melanocyte-stimulating hormone (MSH) can inhibit the growth of cultured melanoma cells [1, 2]. It is not clear whether this growth inhibition is due to the ability of MSH to increase intracellular cyclic AMP levels [3, 4] or due to the accumulation of cytotoxic intermediates of melanin biosynthesis [5]. Analogs of cyclic AMP have been demonstrated to alter melanoma cell morphology [2], inhibit cell replication [6], and stimulate melanogenesis [7]. The relationship between MSH and cyclic AMP and the effects they have on cultured melanoma cells has been the subject of intensive study and debate.

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We have previously reported that B-16 mouse melanoma metastatic variants differ markedly in control of melanogenesis [8] and regulation of cyclic AMP metabolism [9]. In an attempt to determine the relationship among melanogenesis, cyclic AMP metabolism, and metastatic potential we isolated variants of B-16 F_1 (low metastatic potential) mouse melanoma cells which were resistant to growth inhibition by MSH. We report here some of the properties of these resistant cells and their biochemical alterations which enable them to replicate in the presence of MSH.

MATERIALS AND METHODS

Isolation of Cells Resistant to MSH Growth Inhibition

B-16 F_1 (low metastatic potential) mouse melanoma cells were obtained through the courtesy of Dr. I. J. Fidler, Frederick Cancer Research Laboratories, Frederick, Maryland. Stock cultures of these cells were routinely maintained in minimal essential medium (MEM) containing Earle's salts, nonessential amino acids, vitamin solution, L-glutamine (2 mM), sodium pyruvate (1 mM), 50 μg/ml streptomycin sulfate, 50 units/ml penicillin G + 10% heat-inactivated fetal bovine serum (Gibco), adjusted to a final pH of 7.4 and were grown in a 37°C, 95% air–5% CO_2 humidified incubator.

Unfortunately we were not successful in achieving a one-step selection procedure for the isolation of resistant cells. Therefore a sequential two-step procedure was followed. We seeded 1×10^5 B-16 F_1 cells into T-25 flasks. One day later, the medium was aspirated and the flasks were refed with medium containing 0.2 μg/ml MSH. After 7 days in this medium (refeeding every other day) the surviving cells were seeded into another T-25 and grown in the presence of 2 μg/ml MSH for 7 days. After this time the cells were prepared for cloning by the seeding of 100 viable cells onto 60-mm dishes with medium containing 2 μg/ml MSH. After allowance of 2 weeks for colony development ten individual clones were isolated using stainless steel cloning cylinders.

Cell Growth Measurements

Various clones or parental cells were seeded at 1.0×10^5/60-mm dish. One day later cells were refed with either unsupplemented medium or medium containing 0.2 μg/ml MSH. In some experiments 0.5 mM 8-bromocyclic AMP (8BrA) + 0.2 mM 1-methyl-3-isobutylxanthine (MIX) was also added to the culture medium. At 24, 48, and 72 hours after addition of these compounds, triplicate plates were processed for cell counts with a model B Coulter Counter.

Tyrosinase Assay

Parental or variant cells were seeded at $(0.4–3.0) \times 10^5$/100-mm dish. One day after seeding all plates were refed with complete medium ± 0.2 μg/ml MSH. Twenty-four hours after this refeeding tyrosinase was measured as described previously [8].

Protein Kinase Assay

Kinase activity was measured by the assay of Corbin et al [10]. Mouse melanoma parental and variant cells were grown to confluence on 100-mm culture dishes. We prepared cells for assay by washing the monolayer three times with 4 ml of 0.05 M phosphate buffer (pH 6.8), scraping the cells from the dishes ($\sim 4 \times 10^6$/dish) with a rubber policeman, and sonicating the resultant suspension (1.5 ml) for 30 seconds at setting #3 in a model WI85 sonifer (Heat Systems, Plainview, New York). The reaction mixture consisted of 0.05 M phosphate buffer (pH 6.8), 0.2 mM γ-^{32}P ATP (180 cpm/pmole), 0.5 mg histone (type II-

A) ± various concentrations of cyclic AMP, and 20 μl of cell homogenate. Following a 10-minute incubation at 30°C, 50 μl of the reaction mixture was pipetted onto Whatman 3-MM filter paper disks (2.3-mm diameter; Whatman, Inc., Clifton, New Jersey). The disks were dropped into ice-cold 10% trichloroacetic acid (TCA) and washed in sequence with 10% TCA, 95% ethanol, and ether. After drying, the filters were placed in scintillation vials containing Aquasol (New England Nuclear, Boston, Massachusetts) and counted. All experimental samples were corrected for endogenous phosphorylation, ie, the amount of phosphorylation in the absence of histone or the absence of cyclic AMP.

Cyclic AMP Measurements

Parental and variant mouse melanoma cells were prepared and assayed for the ability of MSH to increase intracellular cyclic AMP levels by previously described techniques [9], with the exception that 35-mm dishes were employed and cell densities at the time of stimulation were much lower than previously reported.

Assay of Tumor Growth

Viable cells (1×10^5) of F_1 and variant lines were injected subcutaneously into C57/Bl mice – ten mice for each cell type. Mice were checked every other day for appearance of tumors and the time at which tumors became palpable was recorded. Tumor development in each individual mouse was then followed and the time required to kill the host was noted.

RESULTS

Following isolation of clones from the parental B-16 F_1 cells, they were tested for growth in the absence and presence of MSH. Only 4 of 10 clones showed significant resistance to MSH-induced growth inhibition. One clone (#4) was completely unaffected by MSH even after 72 h of treatment. This particular clone was utilized for further investigation and was given the designation B-16-F_1-MR-4.

F_1 and MR-4 cells were tested for growth in the presence of MSH or 8-BrcA + MIX (Fig. 1). F_1 cells were found to be growth-inhibited by both MSH and 8-BrcA + MIX, although the cyclic AMP analog was more effective. The MR-4 clone differed from F_1 in several aspects. First, the growth rate of untreated MR-4 cells was greater than control F_1 cells. Second, MR-4 cells treated with MSH were not growth-inhibited, while MR-4 cells treated with the cyclic AMP analog were significantly inhibited at all time points. In summary, F_1 was growth-inhibited by both MSH and cyclic AMP, the latter being more effective; MR-4 had a higher replication rate than F_1, was completely resistant to inhibition by MSH, but was susceptible to inhibition by exogenously supplied cyclic AMP.

We suspected that one reason why MR-4 was resistant to MSH-induced growth inhibition was an alteration in tyrosinase, the key enzyme in melanin biosynthesis, since Pawelek et al [2] found that variants of S91 mouse molanoma cells resistant to MSH growth inhibition invariably had altered tyrosinase activities. Therefore, we measured tyrosinase activity in F_1 and MR-4 cells that had been incubated in the absence or presence of MSH for 24 h. Under our assay conditions, the enzyme activity from both cell lines was linear with respect to time and protein concentration. The data (Table I) clearly show that there was a marked reduction of MR-4 basal tyrosinase activity, ie, the activity present in the absence of MSH. Also, MSH was able to stimulate tyrosinase activity approximately threefold in F_1, while only a twofold stimulation was achieved in MR-4, and the absolute levels of hormonally induced tyrosinase activity were six times less in MR-4 than in F_1.

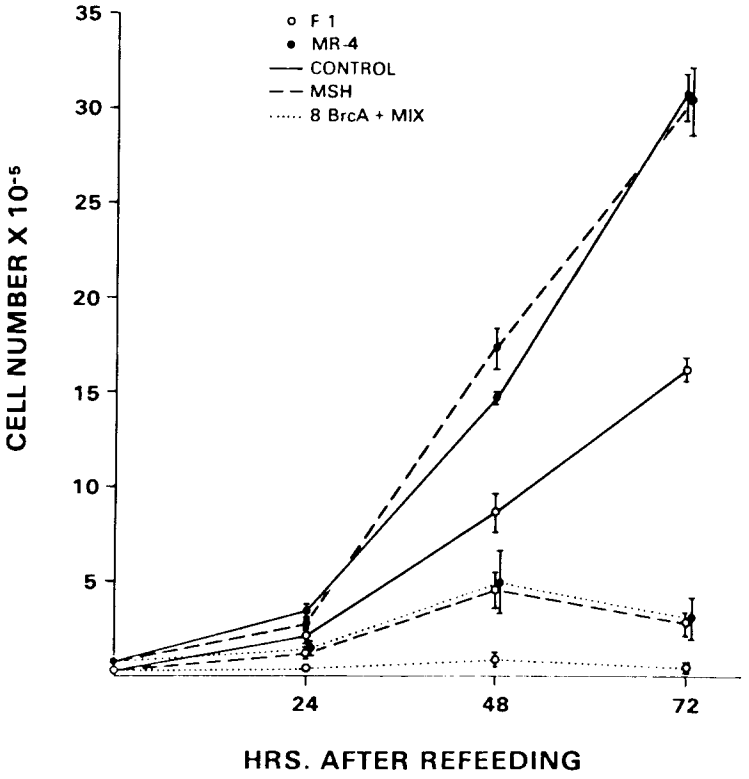

Fig. 1. Growth of F_1 and MR-4 cells in the absence or presence of MSH or 8-bromocyclic AMP + 1-methyl-3-isobutylxanthine. Cells were seeded at 1×10^5/60-mm culture dish. One day later the cells were refed with complete medium ± 0.2 μg/ml MSH, or ± 5×10^{-4} M 8-bromocyclic AMP + 2×10^{-4} M 1-methyl-3-isobutylxanthine. At 24, 48, and 72 h after refeeding, plates were processed for cell counts. The data are represented as the mean ± SEM (bars above and below the data points) of triplicate plates.

Since we had shown that MR-4 cells were still inhibited by the exogenous addition of cyclic AMP, there existed the possibility that MSH failed to inhibit growth because of receptor-adenylate cyclase dysfunctions and hence insufficient production of intracellular cyclic AMP. Therefore we measured the ability of MSH to increase cyclic AMP levels in intact F_1 and MR-4 cells.

The data in Table II clearly show that at any time point measured MR-4 cells produced more cyclic AMP in response to MSH than did F_1 cells. Therefore it is unlikely that the failure of MR-4 cells to be inhibited by MSH is due to a defect in the MSH-receptor-adenylate cyclase complex.

Since it appears that the only mechanism through which cyclic AMP exerts its physiologic actions is the activation of cyclic AMP-dependent protein kinase, we decided to examine the possibility that changes in protein kinase activity might be responsible for MR-4 resistance to MSH. Both F_1 and MR-4 were assayed for protein kinase activity using histone as the phosphate acceptor and using various concentrations of cyclic AMP to stimulate the enzyme. Our assay conditions resulted in linearity with respect to time and protein concentration for the kinases from each cell line. It is apparent from the data (Table III and Fig. 2) that the protein kinase activity of MR-4 differs substantially from that of F_1. The specific activity of the MR-4 kinase under all four assay conditions (Table III)

TABLE I. Tyrosinase Activity in F_1 and MR-4 Cells

Cell type	Specific activity
F_1	8.6
F_1 + 0.2 μg/ml MSH	26.5
MR-4	2.0
MR-4 + 0.2 μg/ml MSH	4.1

Cells were prepared and assayed for tyrosinase activity as described in Materials and Methods. Specific activity is defined as nanomoles of tyrosine hydroxylated per 10^6 cells per hour. Cell densities at the time of the experiment were as follows: F_1 control, 3.4×10^6; MR-4 control, 4.8×10^6; F_1-MSH, 2.4×10^6; MR-4-MSH, 4.4×10^6. The experiment was repeated two additional times with similar results.

TABLE II. Effect of MSH on Cyclic AMP Levels in Intact F_1 and MR-4 Cells

Cell type	Incubation time (min)	Picomoles cyclic AMP/10^5 cells
F_1	5	161 ± 24
	15	393 ± 36
	30	326 ± 12
	60	302 ± 49
MR-4	5	729 ± 72
	15	1,698 ± 37
	30	814 ± 182
	60	443 ± 52

Cells were prepared for hormone stimulation and cyclic AMP was extracted and quantitated as described in Materials and Methods. MSH was present at a concentration of 0.2 μg/ml. Control levels of cyclic AMP at all time points were 0.7 ± 0.2 pm/10^5 cells (F_1) and 1.2 ± 0.3 pm/10^5 cells (MR-4). The data are represented as the mean ± SEM of triplicate plates, each assayed in duplicate in the radioimmunoassay determination for cyclic AMP levels.

TABLE III. Protein Kinase Activity in F_1 and MR-4 Cells

	Picomoles ^{32}P transferred to histone/10^6 cells	
Reaction mixture	F_1	MR-4
−cyclic AMP, −histone	92.0	40.6
+cyclic AMP, −histone	160.7	48.6
−cyclic AMP, +histone	305.1	60.4
+cyclic AMP, +histone	635.2	127.4

Cells were prepared and assayed for protein kinase activity as described in Materials and Methods. The concentration of cyclic AMP used in this experiment was 2 μM. The entire experiment was repeated three additional times with similar results. Cell numbers at the time of the experiment were 2.8×10^5 (F_1) and 4.0×10^5 (MR-4).

was markedly depressed relative to F_1. Also the MR-4 protein kinase was almost maximally stimulated by low concentrations of cyclic AMP (10^{-8} M), while a similar enzyme preparation from F_1 required 10^{-6} M cyclic AMP to be maximally stimulated. At the point of optimal stimulation of protein kinase in both cell lines, the specific activity of F_1 was still 2.8 times higher than the specific activity of MR-4 (Fig. 2).

We next compared the biologic activity (tumor formation) of F_1 and MR-4 cells. When an equal number of viable cells were injected subcutaneously into C57/B1 mice, it was observed that the MR-4 cells formed palpable tumors earlier (an average of 5 days vs 13 days) and killed their host sooner (an average of 14 days vs 22 days after appearance of tumor) than did F_1 cells (Table IV).

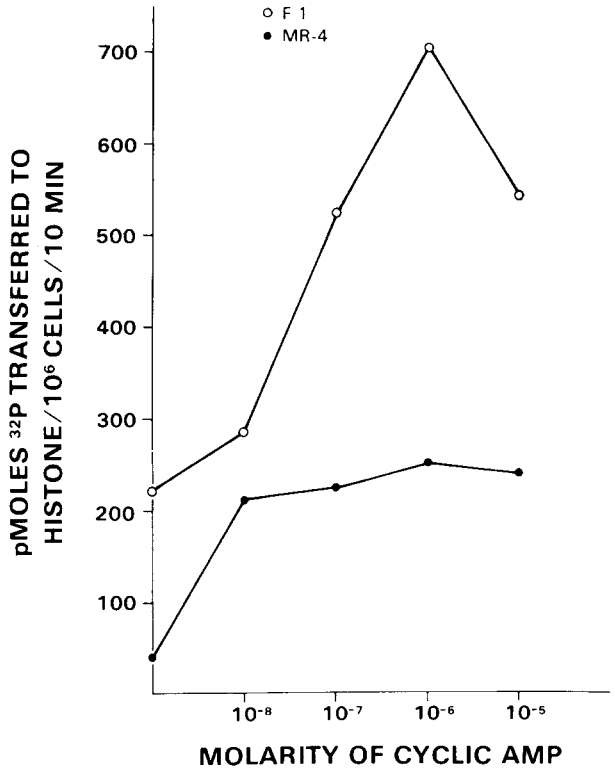

Fig. 2. Protein kinase activity in F_1 and MR-4 cell homogenates as a function of cyclic AMP concentration. Cells were prepared and assayed for protein kinase activity as described in Materials and Methods. Specific activity is expressed as picomoles of ^{32}P transferred from γ-^{32}P ATP to histone per 10^6 cells. Cell densities at the time of assay were 6.3×10^6 (F_1) and 6.1×10^6 (MR-4). The experiment was repeated three additional times with similar results. The initial points on the y axis represent the enzyme activity in the absence of any added cyclic AMP.

TABLE IV. Tumorogenicity of F_1 and MR-4 Cells

	Latency period (days)		Survival time (days)	
Cell type	Range	Ave	Range	Ave
F_1	9–18	13	12–28	22
MR-4	4–6	5	10–18	14

The protocol for assaying tumorogenicity is described in Materials and Methods. Latency period is the length of time before the appearance of a palpable mass. Survival time is defined as the period of time the mouse survived following the appearance of the tumor. There were ten mice in each treatment group.

DISCUSSION

The results presented in this communication suggest that the biochemical alterations which allow MR-4 cells to grow in the presence of MSH are markedly lower levels of tyrosinase and cyclic AMP-dependent protein kinase activities. Pawelek et al [2] have also found depressed tyrosinase activity in two mutants of Cloudman S91 melanoma cells selected for their ability to replicate in the presence of MSH. However, in contrast to their results, we found that MR-4 cells were markedly inhibited by the exogenous addition of 8BrcA to the culture media. Also unlike our MR-4 cells, the Cloudman S91 melanoma mutants did not have depressed cyclic AMP-dependent protein kinase activity.

The mechanism whereby MSH inhibits cell replication has been the subject of controversy. Some feel that the cytostatic action of MSH is due to its ability to increase intracellular cyclic AMP, high levels of which have been demonstrated to be a negative regulator of growth in many types of cultured cells [11–13]. Other investigators believe that MSH indirectly inhibits growth by its stimulation of melanin biosynthesis, the intermediates of which are known to be cytotoxic [5, 14–16]. On the basis of our results we would argue that both groups are partially correct. It is obvious that the markedly lower tyrosinase activity in MR-4 even in the presence of MSH leads to a much lower rate of melanin production and hence a noncytotoxic level of intermediates. In fact when parental F_1 cells are grown on tyrosine-free medium MSH no longer inhibits cell proliferation (data not shown). On the other hand, although MR-4 cells are resistant to MSH, they are still growth-inhibited by cyclic AMP, although not to the same extent as F_1 cells. This may be the result of a *persistent* activation of protein kinase, leading to an imbalance of phosphorylation-dephosphorylation reactions which may be necessary for replication. Alternatively the cyclic AMP may be metabolized to a cytotoxic product by the cells, although this possibility was not likely in our experiments since the potent phosphodiesterase inhibitor MIX was included in the incubation mixture.

The mechanism responsible for depressed protein kinase activity in MR-4 cells is currently under investigation. Among the possibilities are decreased synthesis of holoenzyme, increased turnover of holoenzymes, increased production of regulatory subunits or decreased production of catalytic subunits, and increased production of protein kinase inhibitors. It should be noted that kinase activity in MR-4 was depressed relative to that in F_1 under all four assay conditions (no cAMP, no histone; +cAMP, –histone; –cAMP, +histone; and +cAMP, +histone). From these data it could be argued that the alteration affects the holoenzyme rather than one or the other subunits.

There is convincing evidence that cyclic AMP is the intracellular mediator of MSH-induced tyrosinase activity and hence melanogenesis [3, 4, 7, 17]. Since the only known mechanism through which cyclic AMP exerts its physiologic response is the activation of protein kinase [18, 19], it is presumed that phosphorylation of specific proteins is the mechanism through which MSH increases tyrosinase activity [20, 21]. In light of the biochemical alterations we have demonstrated in MR-4, namely, depressed tyrosinase and protein kinase activity, it is tempting to speculate that in the B-16 mouse melanoma system *total* tyrosinase activity, ie, the activity present in either the absence or presence of MSH, is regulated by cyclic AMP-dependent protein kinase. This hypothesis is strengthened by our recent observations that another variant of F_1 selected for enhanced pulmonary metastatic potential in vivo (B-16-F_{10}) has markedly depressed tyrosinase activity relative to F_1 [8] and also has significantly lower protein kinase activity (Niles and Logue, unpublished observations). This hypothesis could be tested by obtaining a variant of F_1 which had completely defective or absent protein kinase. Such cells should have no detectable levels of tyrosinase activity and hence would be amelanotic melanomas.

The mechanism whereby MR-4 cells accumulate more cyclic AMP in response to MSH than do F_1 cells is currently under investigation. However, it is interesting to note that Bourne et al [19] found that mutants of S49 lymphoma cells resistant to dibutyryl cyclic AMP accumulated significantly more cyclic AMP in response to catecholamines, prostaglandins, and cholera toxin than did wild-type cells.

Our original objective in obtaining F_1 variants resistant to MSH-induced growth inhibition was to obtain cells with altered cyclic AMP metabolism. Using this approach, we hope to determine what role the altered cyclic AMP metabolism we have previously observed for F_{10} cells [8, 9] plays in their enhanced metastatic potential. The data we have acquired concerning the biologic behavior of MR-4 indicate that they have increased malignant potential relative to F_1 cells. It is possible that the ability of the MR-4 cells to resist MSH-induced growth inhibition and hence grow more rapidly than F_1 in vitro may account for their greater rate of growth in vivo. In cooperation with Dr. I. J. Fidler of the Frederick Cancer Research Center, a comparative study of the pulmonary metastatic potential of F_1 and MR-4 is currently in progress. Thus it appears that the selective alteration of a few biochemical parameters can result in a progression of malignancy.

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REFERENCES

1. Wong G, Pawelek J: Nature New Biol 241:213, 1973.
2. Pawelek J, Sansone M, Koch N, Christie G, Halaban R, Hendee J, Lerner AB, Varga JM: Proc Natl Acad Sci USA 72:951, 1975.
3. Pawelek J, Wong G, Sansone M, Morowitz J: Yale J Biol Med 46:337, 1973.
4. Kreiner PW, Gold CJ, Keirns JJ, Brock WA, Bitensky MW: Yale J Biol Med 46:583, 1973.
5. Graham DG, Tiffany SM, Vogel FS: J Invest Dermatol 70:113, 1978.
6. Dipasquale A, McGuire J: J Cell Physiol 93:395, 1977.
7. Johnson GS, Pastan J: Nature New Biol 237:267, 1972.
8. Niles RM, Makarski J: J Natl Cancer Inst 61:523, 1978.
9. Niles RM, Makarski J: J Cell Physiol 96:355, 1978.
10. Corbin JD, Keely SL, Park CR: J Biol Chem 250:218, 1975.
11. Sheppard JR: Proc Natl Acad Sci USA 68:1316, 1971.
12. Johnson GS, Friedman RM, Pastan IH: Proc Natl Acad Sci USA 68:425, 1971.
13. Pastan IH, Johnson GS, Anderson WB: Annu Rev Biochem 44:491, 1975.
14. Lerner AB: Am J Med 51:141, 1971.
15. Halaban R, Lerner AB: Exp Cell Res 108:111, 1977.
16. Halaban R, Lerner AB: Exp Cell Res 108:119, 1977.
17. Kreider JW, Wade DR, Rosenthal M, Densley T: J Natl Cancer Inst 54:1457, 1975.
18. Kuo JF, Greengard P: Proc Natl Acad Sci USA 64:1349, 1969.
19. Bourne HR, Coffino P, Tompkins GM: J Cell Physiol 85:611, 1975.
20. Wong G, Pawelek J: Nature 255:644, 1975.
21. Korner A, Pawelek J: Nature 267:444, 1977.