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Anchorage-independent Growth of Murine Melanoma in Serum-less Media Is Dependent on Insulin or Melanocyte-stimulating Hormone

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α-Melanocyte-stimulating hormone (MSH) is known to stimulate melanogenesis in murine melanoma, particularly in Cloudman S-91 melanoma cells. The effects of MSH and insulin on the proliferation of S91 murine melanoma cells have aroused controversy; in various reports, both hormones have been reported to either stimulate or inhibit murine melanoma growth. In our studies both MSH and insulin stimulated the colony-forming ability and the proliferative capacity of S-91 murine melanoma cells grown in soft agar with either serum-supplemented or serum-less medium. Unless insulin and/or MSH were present, Cloudman S-91 melanoma cells failed to clone in soft agar. The insulin effect was greater than that of MSH, and was more pronounced in serum-less than in serum-supplemented medium. The concurrent treatment of S91 melanoma cells with both MSH and insulin resulted in a greater increase in the total number of colonies formed than caused by treatment with either hormone alone. The combined MSH-insulin stimulation of anchorage-independent growth was specific, since the effect could not be mimicked by epidermal growth factor (EGF), gonadotropin-releasing hormone (GRH), luteinizing hormone (LH), nerve growth factor (NGF) or platelet-derived growth factor (PDGF). Therefore, MSH and insulin may be specific growth factors for murine melanoma cells. © 1985 Academic Press, Inc.

MSH (α-melanocyte stimulating hormone) induces melanogenesis [1, 2], and under certain conditions stimulates the anchorage-dependent growth of murine melanoma [3]. However, it has also been reported that MSH retards the growth of Cloudman S-91 murine melanoma cells [2]. Halaban & Lerner [3] suggested that this inhibition was due to culture conditions, since it was only evident 6 days after the initial exposure to MSH. When MSH was injected into tumor-bearing animals, it either had no effect [1] or else stimulated tumor growth [4]. The in vitro inhibitory effect of MSH on cellular proliferation observed by some investigators may be attributed to the presence of hormones such as adrenocorticotropic hormone (ACTH) and corticosterone [5] in fetal and bovine serum [6]. Therefore, in order to determine the true response of murine melanoma cells in culture to a hormone it is appropriate to use serum-less media to avoid the effects of other hormones which are incidentally present in serum.

We report here the effect of MSH and other hormones on anchorage-indepen-

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dent growth of S-91 murine melanoma in serum-less media. In this study we used a bilayer soft agar assay to measure the effect of hormones on anchorage-independent growth [7]. Colony formation in agar may be the best in vitro assay for transformation [8, 9], and has been used to study the biology of human tumor cells as well as to estimate the chemosensitivity of human tumor cells to new and established drugs, as determined by their ability to inhibit colony growth [10–15]. Recently this system has been used to study the effect of biological modifiers on the clonogenic growth of human melanoma [16–19]. The data presented in this study indicate that the anchorage-independent growth of murine melanoma colony-forming cells (MCFU) was dependent on insulin and/or MSH, and support the contention that insulin and MSH-like peptides might function as growth factors in supporting the growth of transformed murine melanoma cells.

MATERIALS AND METHODS

Hormones

Synthetic gonadotropin-releasing hormone and synthetic MSH, and bovine serum albumin (Cohn fraction V) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Insulin, Iletin I, was obtained from Eli Lilly and Co. (Indianapolis, Ind.). Epidermal growth factor (EGF), nerve growth factor (NGF) and platelet derived-growth factor (PDGF) was obtained from Collaborative Research (Lexington, Mass.). [Nle^4, D-phe^7]-α-MSH was a gift from Vega Biochemical (Tucson, Ariz.). Radioactive [ring-3,5-^{3}H]L-tyrosine (sp. act. 48 Ci/mole) was obtained from New England Nuclear (Boston, Mass.).

Media

Fetal bovine (FCS) and horse serum (HS) were purchased from K.C. Biological (Lenexa, Kans.). Ham's F-10 and Dulbecco's Modified Eagle media (DMEM), L-glutamine and penicillin/streptomycin solution were obtained from Gibco Laboratories (Santa Clara, Calif.). Stock cultures were maintained in both serum and serum-less media. Serum-based medium consisted of either Ham's F-10 or DMEM fortified with biotin (0.1 μg/l), vitamin B-12 (1.3 μg/l) and coenzyme B-12 (1 μg/l). Both media were supplemented with 10% HS and 2% FCS which had been heat-inactivated. Serum-less medium consisted of fortified DMEM supplemented with 1% BSA and 2 mM glutamine. All media contained 100 IU penicillin and 100 μg of streptomycin per ml.

Cells

The Cloudman S-91 CCL 53.1 murine melanoma cell line was obtained from the American Type Culture Collection Repository. The melanotic (Mel 11A) variant was cloned from CCL 53.1 and obtained from M. Hadley (Molecular and Cellular Biology, Department, University of Arizona). Our laboratory has carried these two lines by a combination of passage through DBA/2J mice followed by limited sub-culturing on plastic. One million cells injected subcutaneously into a mouse formed a tumor in 2 weeks and the phenotype was maintained in vivo. Tumors were excised, minced and passed through a series of needles (18 and 22 gauge) to form a single-cell suspension. Cells were then plated in plastic culture flasks and cultured through one passage to increase the cell yield. Several aliquots were frozen in serum-based medium containing 10% dimethylsulfoxide (DMSO) and stored under the vapor phase of liquid nitrogen. Cells were recovered from liquid nitrogen by rapid thawing, plated and allowed to adhere to the plastic in serum-based medium for 24 h. Half the cells were changed to serum-less medium and allowed to grow through one or two passages before being used in experimental procedures. Stock cultures were routinely cultured no more than ten 7-day passages before replacement with low passage cells from frozen stock.

Soft Agar Assay

Five thousand cells (unless stated otherwise) and the appropriate hormone additives were plated in 0.3% agar in medium in 30 mm diameter Falcon Petri dishes over an underlayer of medium of 0.5% agar. The same medium was used for the top and bottom layers. After 10 days the colonies were counted and sized by diameter using an optical image analyser (FASII Bausch & Lomb, Rochester, N.Y.) as previously described [7]. Using a nomogram which we have recently constructed in our laboratory, the total number of cells in a colony could be determined from the cell and colony diameter [13]. This procedure enabled us to determine that melanoma cell growth units (MCFU) greater than 42 μm in diameter contained at least 10 cells, those greater than 60 μm in diameter contained at least 28 cells, those greater than 104 μm in diameter contained at least 105 cells and those greater than 149 μm in diameter contained 247 cells [13, 14].

Tyrosinase Assay

Tyrosinase activity was assayed by a modification of the method of Pomerantz [20, 21]. 2×10^5 cells grown in serum-supplemented Ham's F-10 medium were seeded into 25 cm^2 flasks. Forty-eight hours after seeding, the medium was changed and the cells were treated with the appropriate hormone concentrations. On the following day, the medium was replaced with medium containing 1 μCi/ml [^{3}H]tyrosine and the cells were treated for another 24 h with hormones. At the end of this 24-h period, the cells were harvested and counted and the labeled medium assayed for tyrosinase activity. One-ml aliquots of medium were each treated with 1 ml of activated charcoal (10% w/v in 0.2 N citric acid). Charcoal was removed by centrifugation at 2500 rpm for 15 min. One-ml aliquots of the supernatant were then passed over Dowex 50 resin columns equilibrated with 0.1 N citric acid to remove any residual [^{3}H]tyrosine. Columns were rinsed with 1 ml of 0.1 N citric acid and the eluant was collected directly into scintillation vials.

RESULTS

Growth of Murine Melanoma Cells in Serum-containing and Serum-less Media

The MCFU in the Cloudman S-91 CCL 53.1 line have a very high plating efficiency in soft agar [14, 22]. 60% of the cells readily formed colonies in serum-based medium (table 1). The clonogenic cells also had a high proliferative capacity with 60% of the growth units reaching a diameter greater than 149 μm by day 10. From sizing experiments it was determined that this class of growth unit contained more than 247 cells, representing eight doublings [13]. Since a linear relationship exists between the initial number of cells plated and the number of colonies formed in soft agar, plating of murine melanoma cells at near optimal cell density [14] leaves adequate room for expression of the transformed phenotype and proliferative potential.

Unlike serum-supplemented media, serum-less media did not support colony formation in soft agar (table 1). Most of the CCL 53.1 murine melanoma cells failed to divide even once after 20 days. Serum-less media did not support the anchorage-independent growth of CCL 53.1 murine melanoma cells without altering other biological parameters. Subculturing the murine melanoma cells as a monolayer on plastic in DMEM-based serum-less media for 9 weeks did not alter their morphology or tumorigenicity. Ten DBA/2J mice were each given two injections of 1×10^6 CCL 53.1 cells which had been subcultured for 5 weeks in serum-less media. All the injection sites yielded a tumor in 2 weeks. As shown in

table 2, MSH stimulated tyrosinase activity of cells grown in serum-less or serum-supplemented Ham's F-10 medium. Interestingly, the basal tyrosinase levels of both CCL 53.1 and MEL 11A cells were lower in the serum-less than in the serum-supplemented medium, suggesting that serum contains factors with MSH-like activity which need not be MSH-like with respect to their mechanism of action.

Effect of MSH on Anchorage-independent Growth

In serum-less media, treatment of CCL 53.1 cells in soft agar with MSH resulted in a 2–4-fold increase in colonies by day 10 (table 1). Unlike the control cells that failed to divide many of the MSH-treated murine melanoma cells underwent one or two divisions. Growth units down to 42 μm in diameter were measured and quantitated in experiment 2 (table 1). No additional growth units were observed in the control plates, but in the MSH-treated plates there was a 4-fold increase in the number of growth units greater than 42 μm in diameter.

Table 1. *Insulin and MSH stimulate the anchorage-independent growth of CCL 53.1 cells in serum-less medium*

Growth conditions		Growth units (diameter) >42 μm	>60 μm	>104 μm	>149 μm
Serum		–	3 000±17	1 912±120	1 880±90
Serum-less					
Experiment 1					
Control		–	2±2	0	0
EGF	1–10 ng/ml				
GRF	1–50 ng/ml				
LH	1–50 ng/ml	(All)	2±2	0	0
NGF	1–50 ng/ml				
PDGF	1–50 ng/ml				
Insulin	1.5×10^{-9} M	–	238±106	62±26	10±3
	1.5×10^{-8} M	–	1 424±164	1 044±176	526±118
	1.5×10^{-7} M	–	1 956±36	1 340±26	518±21
MSH	1.0×10^{-7} M	–	8±1	0	0
+ Insulin	1.5×10^{-9} M	–	782±130	168±72	26±9
+	1.5×10^{-8} M	–	2 120±120	1 258±88	366±24
+	1.5×10^{-7} M	–	1 936±28	1 456±34	656±19
MSH	1.0×10^{-8} M		6±3	0	0
+ Insulin	1.5×10^{-9} M		1 990±180	1 064±136	136±58
+	1.5×10^{-8} M		2 360±25	1 856±38	930±29
Experiment 2					
Control		4±2	4±2	0	0
MSH 1×10^{-7} M		66±8	16±3	0	0
Insulin 1.5×10^{-9} M		972±29	742±44	266±42	
Insulin+MSH		2 572±38	1 928±26	588±34	

MCFU also responded to MSH *in serum* (table 3). At low cell density (625 cells/plate) MSH caused an 8-fold increase in the number of growth units reaching 42 μm in diameter. MSH also produced a large increase in the proliferative capacity of MCFU grown in serum, which was evidenced by the increase in the number of large colonies. Each of the control plates had a total of 48 growth units that were greater than 42 μm in diameter, while the MSH-treated plates had 308 growth units which were greater than 104 μm in diameter. The MCFU in the control plates underwent an average of three doublings, while the growth units greater than 104 μm present in the MSH-treated plates underwent seven doublings. The promoting effects of MSH on colony formation and size were discernible, but not as remarkable at high cell densities. [Nle^4, D-Phe^7]-α-MSH, a potent MSH analogue [23], was slightly more effective than MSH in inducing anchorage-independent growth.

MSH was even more effective in inducing the highly melanotic Mel 11A cells to initiate growth in agar and form colonies in serum-less media (table 4). The addition of MSH caused an 8-fold increase in the number of colonies formed. The initiation of anchorage-independent growth was MSH dose-dependent as evident by a 50% increase in colony number at the higher hormone concentration. [Nle^4, D-Phe^7]-α-MSH at 10^{-8}M was more effective than MSH in inducing Mel 11A MCFU to form colonies (table 4).

Effect of Insulin on Anchorage-independent Growth

Insulin was more potent than MSH in initiating and promoting colony formation in agar (tables 1, 4). A nanomolar dose of insulin was sufficient to increase

Table 2. *Tyrosinase activity of murine melanoma in serum and serum-less medium*

	CPM/10^6 cells
CCL 53.1	
Serum	
Control	6 500±650
MSH 10^{-7} M	21 042±1 450
Serum-less	
Control	638±255
MSH 10^{-7} M	27 043±1 777
Mel 11A	
Serum	
Control	52 380±1 800
MSH 10^{-7} M	111 190±5 400
Serum-less	
Control	34 060±3 200
MSH 10^{-7} M	108 486±3 100

the number of CCL 53.1 colonies formed in serum-less medium by 100- to 200-fold. This stimulation was insulin dose-dependent. Insulin also enhanced the proliferative capacity (colony size) of these CCL 53.1 colony-forming cells, exemplified by the presence of 500 growth units greater than 149 μm in diameter in plates treated with 10 nM insulin, compared with absence of such large colonies in control plates (table 1). Insulin also modulated colony growth in serum, since the addition of sub-nanomolar insulin to serum-supplemented cultures caused a 33% increase in the number of colonies formed by day 6 (table 5). The experiment had to be terminated by day 6, because the insulin-treated cells grew so fast that the medium was depleted by this time. Increasing the insulin concentration had no effect on the number of growth units reaching colony size; however, there was a dose-independent increase in the size of the colonies.

Effect of Combined MSH and Insulin on Anchorage-independent Growth

The concomitant addition of insulin and MSH to CCL 53.1 cells plated in soft agar with serumless media resulted in a larger increase in the number of colonies formed over that seen with either insulin or MSH alone (table 4). The addition of both MSH and insulin effected an almost 3-fold increase in the number of colonies and a 2½-fold increase in the number of growth units reaching at least 104 μm in diameter over that seen with nanomolar insulin alone. The stimulation of colony formation was more pronounced at low insulin concentrations. Mel 11A cells were even more responsive to the insulin–MSH combination, which elicited a 5-fold increase in the number of colonies formed over that seen with nanomolar insulin alone (table 4). However, with this highly melanotic cell line, the increase in colony number occurred with an appreciable concomitant decrease in colony

Table 3. *MSH stimulates the anchorage-independent growth of CCL 53.1 murine melanoma cells in serum*[a]

	Percent of control growth units (diameter)		
No. of cells plated	>42 μm	>60 μm	>104 μm
625			
Hormone × 10^{-7} M MSH	808	926	1 925
[Nle[4], D-Phe[7]]-α-MSH	921	1 121	2 263
1250			
MSH	209	215	267
[Nle[4], D-Phe[7]]-α-MSH	199	205	261
5000			
MSH	124	123	132
[Nle[4], D-Phe[7]]-α-MSH	134	127	139

[a] DMEM medium containing 10% HS and 2% FCS.

size, especially at the higher insulin concentrations. Specificity of the MSH–insulin effect was demonstrated by the inability of EGF, GRH, LH, NGF and PDGF to elicit colony formation in serum-less medium (table 1).

DISCUSSION

Todaro et al. [24, 25] have put forth the notion that tumor cells produce autologous growth factors which maintain the transformed phenotype. However, murine melanoma does not fit into this scheme. In this report we demonstrated that the anchorage-independent growth of Cloudman S-91 murine MCFU was

Table 4. *Insulin and MSH stimulate colony formation of pigmented mel 11A cells*

	Total growth units (diameter)		
	>60 μm	>104 μm	>149 μm
Control	74	22	0
Insulin			
1.5×10^{-9} M	236	86	14
1.5×10^{-8} M	1 706	1 266	684
1.5×10^{-7} M	1 964	1 514	866
MSH 10^{-7} M	594	56	0
+ insulin 1.5×10^{-9} M	1 032	152	2
+ insulin 1.5×10^{-8} M	2 370	868	236
MSH 10^{-8} M	274	14	0
+ insulin 1.5×10^{-9} M	732	124	6
[Nle^4, D-Phe^7]-α-MSH			
10^{-7} M	528	46	2
10^{-8} M	410	26	0

SE within 8% of the mean value.

Table 5. *Effect of insulin on the anchorage-independent growth of CCL 53.1 murine melanoma cells in DMEM+serum*

	Total growth units (diameter)	
	>60 μm	>104 μm
Control	1 880±90	20
Insulin		
1.5×10^{-10} M	2 712±38	92
1.5×10^{-9} M	2 646±68	216
1.5×10^{-7} M	2 756±88	392

5000 CCL 53.1 cells in agar plated per 2 ml dish and counted on day 6.

stimulated by insulin and MSH, which are not known to be transforming growth factors. There is also a remote possibility that MSH and insulin may be synergistic with factors that may adsorb to BSA, such as small peptides and fatty acids. Normal cells such as hemopoietic and granulosa cells form colonies under appropriate conditions, suggesting that these cells are responding to specific normal growth factors [26–28]. Transforming growth factors have recently been isolated from normal tissues, particularly platelets [29, 30]. Thus, some tumor cells may retain some normal characteristics in that they still respond to tissue-specific hormones. Evidence for this comes from a recent report that the growth of epidermoid tumors, which responded in vitro to EGF, were inhibited by monoclonal antibodies to this factor [31]. The uncontrolled growth may occur because, unlike normal stem cells in which proliferation is arrested after the initial exposure to growth factors, tumor cells retain the activated or transformed state and continue to respond to normal growth factors or other hormones, and retain hormone dependency for their growth and development.

Other workers have reported that the anchorage-dependent growth of Cloudman S-91 murine melanoma was inhibited by insulin [32, 33]. Contrary to these reports, our results show that insulin induced colony formation in soft-agar and stimulated the growth of CCL 53.1 in serum and serum-less media. Mather & Sato [34] had previously reported that insulin was a requirement for the anchorage-dependent growth of melanoma cells in serum-free media. The anchorage-dependent growth of CCL 53.1 was stimulated by insulin, regardless of whether the cells were grown in RPMI 1640 or DMEM (not shown). The discrepancy between our results and those of the other investigators may be related to the different tissue culture media utilized. Kahn et al. [32] and Fuller & Ehlers [33] used Ham's F-10 while we used DMEM. Mather & Sato [34] also used a DMEM-based media in their study. In accordance with the findings of Kahn et al. [32] and Fuller & Ehlers [33], we also found that the anchorage-dependent growth of CCL 53.1 cells was inhibited by insulin in Ham's F-10. Also these murine melanoma cells failed to grow in soft agar which contained serum-less F-10 media, even when insulin and MSH were added. Thus, depending on the medium chosen one can generate completely opposing biological effects. The inhibitory effect of insulin on CCL 53.1 cells when grown in Ham's F-10 may be due to the lower calcium and higher copper, iron and zinc concentrations in this medium. We propose that the behavior of murine melanoma CCL 53.1 cells to insulin in Ham's F-10 medium is not representative of the in vivo situation. However, animal tumor studies will be needed to settle this issue.

Our observation that MSH stimulated the growth of murine melanoma in soft agar is in agreement with a previous report in which MSH stimulated the anchorage-dependent growth of Cloudman S-91 murine melanoma cells [3]. We found that MSH stimulated the anchorage-independent growth of CCL 53.1 cells in serum and serum-less media. Unlike the previously discussed results with insulin, MSH also stimulated the anchorage-independent growth of CCL 53.1

MCFU when they were plated at low cell density in Ham's F-10 and serum (not shown). The increase in colony formation was especially evident in the MSH-treated melanotic Mel 11A plates. However, when Mel 11A cells were plated with insulin plus MSH, a reduction in the number of large colonies was observed. This result agrees with the notion put forth by Halaban & Lerner [35] that tyrosinase-related products cause a concentration-dependent inhibition of proliferation in vitro, which raises the question of whether MSH is a growth-promoting hormone for murine melanoma? At optimal cell densities we observed that MSH-stimulated anchorage-independent growth which is in accordance with the previous observation that MSH did not inhibit the growth of murine melanoma tumors even while it induced an increase in tyrosinase activity [1]. Additional support is provided by Pawelek et al. [4] who reported that MSH stimulated the growth of CCL 53 murine melanoma tumors. These observations indicate the concentration of the by-products of melanin synthesis does not rise high enough in vivo to affect the proliferation of murine melanoma tumors. The soft agar data presented here also suggests that the growth of Cloudman S-91 murine melanoma tumors may be insulin and/or MSH-dependent.

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