vaccines would also skew the benefit-risk ratio of vaccination to the benefit side since the benefit to be derived would be that of immunity against the total number of heterologous pathogens, whereas the risk would be that of the single vaccination.

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18. A 1.8-kb fragment derived from vaccinia Hind III M was directionally cloned into the Hind III–Sal I region of pBR322, generating pMP62. An 800-bp Sph I–Bgl II vaccinia fragment isolated from pMP62 was subjected to partial Rsa I digestion and a 200-bp Rsa I–Bgl II fragment containing an early promoter upstream from the Rsa I site was isolated. The Rsa I terminus and the Sph I site of pMP62 were modified to contain Hind III sites. The Hind III–Bgl II fragment containing an early vaccinia promoter was then ligated into the modified Sph I–Bgl II region in pMP62, generating pMP62-15 and resulting in a deletion of 0.6 kb of vaccinia sequences. The 2.5-kb Hind III–Sst I fragment containing the HSVgD (*5*) coding sequence was trimmed by Nru I digestion (*23*). The 1330-bp fragment was modified by linker addition, and the Hind III–terminated HSVgD coding sequences were inserted into the Hind III site within the vaccinia sequences of pMP62-15, generating plasmid pLP2. This plasmid was used as donor DNA for in vivo recombination (*3*) with vP53, and a novel recombinant vaccinia virus, vP124, containing the HSVgD sequences in addition to the InfHA sequences, was isolated by replica filter plating (*3*).
19. A 2.7-kb Bgl II fragment spanning the vaccinia Hind III M-K junction and containing an internal Bgl II site was inserted into the Bam HI site of pBR322H$^-$, a pBR322 derivative having a deleted Hind III site; this generated pMP18. A 360-bp Hind III–Bam HI fragment containing an early vaccinia virus promoter derived from Hind III fragment I was directionally cloned at the single Hind III–Bgl II site of pMP18, eliminating 0.1 kb of endogenous vaccinia sequences; this generated a novel plasmid pMP18PP. A Hind III–terminated HBsAg coding sequence of 1090 bp (*5*) was inserted at the single Hind III site of pMP18PP in proper orientation with the vaccinia promoter. This donor plasmid pPP18sAg was used as donor DNA for in vivo recombination with vP124 and a novel recombinant vaccinia virus, vP168, containing the HBsAg sequences in addition to the InfHA and HSVgD sequences was isolated by replica filter plating. Plasmid constructions as well as recombinant vaccinia viruses were confirmed by restriction analysis.
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Growth Regulation of Human Melanocytes: Mitogenic Factors in Extracts of Melanoma, Astrocytoma, and Fibroblast Cell Lines

Abstract. *Melanocytes derived from fetal or adult skin do not propagate in vitro unless cultured in the presence of factors such as 12-O-tetradecanoylphorbol 13-acetate (TPA). In a search for physiological factors regulating the growth of melanocytes, extracts of various cultured cell types were tested. Factors produced by melanoma and astrocytoma cell lines support continued proliferation of melanocytes in the absence of TPA. WI-38, a fibroblast cell line derived from human embryonic lung, was the most active source of melanocyte growth factors. No melanocyte growth-promoting activity was found in extracts of cultured neuroblastoma, renal cancer, normal keratinocytes, or renal epithelium. Nerve growth factor, epidermal growth factor, melanocyte-stimulating hormone, transforming growth factor-β, and platelet-derived growth factor did not have growth-promoting activity for melanocytes. The presence of melanocyte growth factors and TPA together resulted in the strongest mitogenic activity for melanocytes, permitting the recovery (at 20 days) of 4 to 20 times as many cells as in growth factor or TPA alone.*

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Melanocytes are the melanin pigment–producing cells of the body, which in normal human skin represent a minor cell population that undergoes mitosis only rarely (*1*). Little is known about melanocyte growth regulation, owing mainly to difficulties in obtaining sufficient numbers of melanocytes for studies in vitro. We showed earlier (*2*) that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) fosters replication of melanocytes in vitro by permitting the preferential attachment of melanocytes from skin cell suspensions and stimulating them to grow. This mitogenic activity of TPA can be potentiated by cholera toxin (*2*) and isobutylmethylxanthine (*3*). In contrast to melanocytes, human melanoma cells generally grow vigorously in vitro in the absence of TPA, suggesting that their independent growth might be associated with the production of melanocyte growth factor or factors. To pursue this idea, we began a search for factors produced by melanoma and other cell types that would permit growth of melanocytes in the absence of TPA.

Melanocytes were isolated and cultured as described (*2*). To avoid fibroblast contamination, we trypsinized the cultures and purified them by immune rosetting and Percoll gradients (*4*), or we treated them with geneticin (*3*). Cultures used for experiments were free of contaminating fibroblasts as shown by leucine-aminopeptidase staining (*5*). Nonpigmented melanoma cell lines were selected for study to avoid the toxic effects of intermediate metabolites in melanin synthesis. In preliminary studies, we evaluated melanocyte growth-stimulating activity in the supernatant fluids of four melanoma cell lines. Since little or no activity was detected, we turned to cell extracts.

Figure 1 illustrates tests with extracts derived from two cell lines of neuroectodermal origin—namely, SK-MEL-131 melanoma and AO_2V_4 astrocytoma—and from the embryonic lung fibroblast line WI-38. Melanocytes cultured in the absence of TPA showed minimal [^{3}H]thymidine incorporation and no cell growth. In the presence of SK-MEL-131, AO_2V_4, or WI-38 extracts, melanocytes showed active [^{3}H]thymidine incorporation and repeated rounds of cell division. Extracts of SK-MEL-131 or AO_2V_4 resulted in a sixfold increase in [^{3}H]thymidine incorporation (as compared to melanocytes grown in the absence of added extract or TPA). Extracts of WI-38 resulted in a 20-fold increase in [^{3}H]thymidine incorporation. With regard to cell growth and division, melanocytes cultured in the absence of TPA generally round up and detach, and after 20 days cultures are lost. The effect of cell extracts on melanocyte growth parallels their effect on [^{3}H]thymidine incorporation, with extracts of WI-38 exerting the strongest mitogenic activity for melanocytes. Titration experiments showed that extracts diluted 1:500 to 1:1000 gave optimal stimulation of melanocyte growth; activity could still be detected at dilutions of 1:10,000. Growth inhibition, most likely nonspecific, was found with extracts diluted less than 1:100. Eighty percent of the growth-stimulating activity of WI-38 extracts was removed

by adsorption with normal melanocytes.

Table 1 summarizes the results of tests with 14 cell extracts. Melanocyte growth-promoting activity was detected in extracts from three melanoma cell lines, a noncultured melanoma specimen, two of four astrocytoma cell lines, and fibroblasts cultured from lung and skin. Extracts from two renal carcinoma cell lines, a neuroblastoma cell line, an embryonic kidney cell strain, and cultured keratinocytes had no melanocyte growth-promoting effect. The following known growth factors were also tested: transforming growth factor-β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), melanocyte stimulating hormone (MSH), and nerve growth factor (NGF) (*6*). No melanocyte growth-promoting activity was detected in cultures supplemented with these factors, either by [^{3}H]thymidine incorporation or by increase in cell numbers.

Exposure of melanocytes to the combined action of TPA and active cell extracts resulted in a synergistic effect on cell growth (Fig. 2). When cultured in either TPA or extract for 20 days, there was a fourfold increase in cell numbers in cultures with TPA and an 8- to 30-fold increase in cultures with extracts. Addition of TPA and extracts together resulted in a 40-fold increase in SK-MEL-131 extracts, a 50-fold increase in AO_2V_4 astrocytoma extracts, and a 90-fold increase in WI-38 extracts. Melanocytes grown under these various conditions showed distinguishing morphologic features. Growth in TPA alone or with added cholera toxin resulted in melanocytes showing a prominent nucleus surrounded by a thin rim of cytoplasm and long dendritic processes (Fig. 2A). Melanocytes grown in extracts were triangular or spindle-shaped, with one or multiple dendrites at each pole (Fig. 2B). Addition of TPA to such cultures caused cell elongation and extension of processes (Fig. 2C). Whereas melanocytes grown in TPA or extracts alone were contact-inhibited, the combined action of TPA and extracts caused the cells to continue to replicate after reaching confluency. Melanocytes cultured in extracts of melanoma, astrocytoma, or WI-38 were more highly pigmented than melanocytes cultured in TPA.

Extracts can also support the growth of melanocytes seeded at low cell densities. Melanocytes (1×10^2 per square centimeter) grew in the presence of extracts, either alone or in combination with TPA, whereas little or no growth was observed when TPA or TPA com-

Table 1. Melanocyte growth-promoting activity of cell extracts. Triplicate cultures were labeled for 4 hours with [^{3}H]thymidine at 72, 96, 120, and 144 hours after the initial addition of cell extracts. The number of [^{3}H]thymidine counts incorporated at each time interval was combined to obtain a total [^{3}H]thymidine count. Comparable studies of [^{3}H]thymidine incorporation were carried out with melanocytes grown in the absence of cell extracts. The stimulation index is given as the ratio of total [^{3}H]thymidine counts of melanocytes grown in the presence of factors to total [^{3}H]thymidine counts of melanocytes grown in the absence of factors. S.E.M., standard error of the mean. See Fig. 1 for determination of cell growth.

Cell extracts	Source	Effect on melanocytes	
		[^{3}H]Thymidine incorporation (stimulation index ± S.E.M.)	Increase in cell number*
SK-MEL-131	Melanoma	6.5 ± 0.30	+
SK-MEL-170	Melanoma	3.6 ± 0.25	+
SK-MEL-178	Melanoma	3.3 ± 0.26	+
Tumor tissue	Melanoma	4.1 ± 0.28	+
AO_2V_4	Astrocytoma	8.3 ± 0.35	+
SK-MG-8	Astrocytoma	3.9 ± 0.30	+
SK-MG-17	Astrocytoma	1.7 ± 0.18	−
SK-MG-12	Astrocytoma	1.2 ± 0.09	−
SK-N-MC	Neuroblastoma	2.6 ± 0.13	−
SK-RC-42	Kidney carcinoma	1.8 ± 0.16	−
SK-RC-28	Kidney carcinoma	1.4 ± 0.07	−
WI-38	Embryonic lung	21.3 ± 2.72	+
FsFb 1143	Foreskin fibroblast	6.7 ± 0.25	+
HEKC	Embryo kidney	1.4 ± 0.09	−
1223 EC	Epidermis	0.8 ± 0.02	−

* − indicates no increase in cell numbers; + indicates one or more population doublings in 20 days.

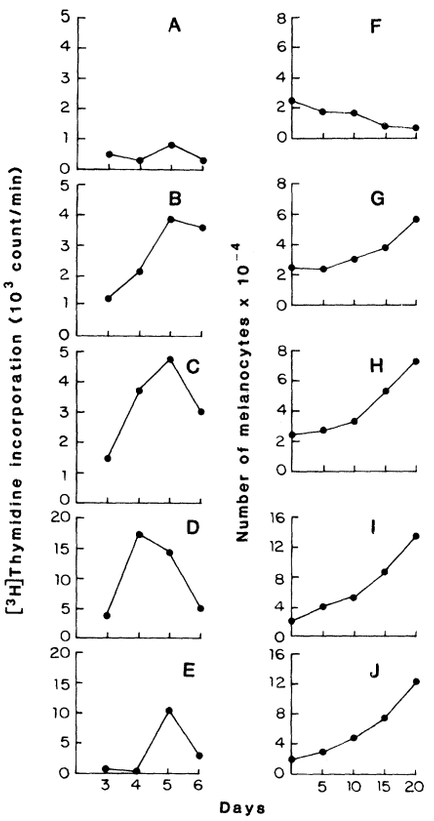

Fig. 1. Melanocyte growth-promoting activity of extracts of SK-MEL-131 melanoma, AO_2V_4 astrocytoma, and WI-38 fibroblasts. (A to E) [^{3}H]Thymidine incorporation. (F to J) Melanocyte cell growth. Melanocytes were maintained in Eagle's minimal essential medium (MEM) with Earle's salts; 0.01 m*M* nonessential amino acids; 2 m*M* L-glutamine containing penicillin (100 unit/ml), streptomycin (0.1 mg/ml), and Fungizone (0.25 μg/ml); and 10 percent fetal bovine serum (cMEM), with the following supplements: (A and F) controls (no supplement); (B and G) SK-MEL-131 extract (1:1000 dilution); (C and H) AO_2V_4 extract (1:1000 dilution); (D and I) WI-38 extract (1:1000 dilution); and (E and J) 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (10 ng/ml). Foreskin melanocyte cultures (designated MC 1217) were grown in cMEM, TPA (10 ng/ml), and cholera toxin (10^{-8}*M*) for 11 passages. They were then seeded in the same medium in tissue culture cluster plates (96 flat-bottom wells) at 5×10^3 per well (A to E) or in tissue culture cluster plates (12 wells) at 2.5×10^4 (E to J). Twenty-four hours later the cultures were washed three times with MEM (not containing TPA and cholera toxin) and incubated for 4 hours. Cells were then fed at 3-day intervals with cMEM containing cell extracts or TPA. Triplicate cultures were labeled for 6 hours with [^{3}H]thymidine (5 μCi/ml) at 72, 96, 120, and 144 hours after the initial addition of TPA or cell extracts. After labeling, the cells were washed three times with phosphate-buffered saline (PBS), dislodged from the wells by trypsin-EDTA solution, and counted in Hydrofluor with a scintillation counter. For evaluation of cell growth, trypsinized cells from triplicate cultures were counted at 5-day intervals. The standard error of the mean was less than 10 percent of the mean counts per minute and cell counts for each point. For the preparation of cell extracts, cultured cells (*8*) were grown to confluency in their respective medium in the presence of 10 percent fetal bovine serum. After thorough washing with PBS, cells were removed by scraping with a rubber policeman, pelleted at 180*g* for 10 minutes, and suspended in PBS (1:1, by volume). All suspensions were sonicated twice for 15 seconds, diluted 1:10, and clarified by two-step centrifugation at 16,000*g* for 20 minutes and 150,000*g* for 45 minutes. Protein content was approximately 1.4 mg/ml as determined by the Lowry procedure (*9*). The supernatants were separated into aliquots and kept frozen at −70°C.

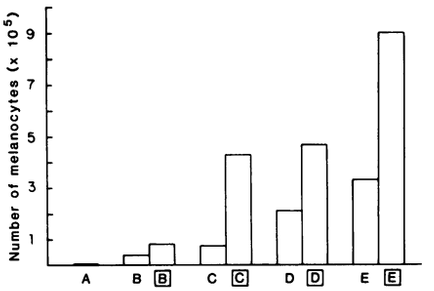

Fig. 2. Influence of added TPA on the melanocyte growth-promoting activity of cell extracts. Melanocytes (see below) were cultured for 20 days in cMEM with the following supplements: A, control (no supplement); B, TPA, and B, TPA plus cholera toxin; C, SK-MEL-131 extract, and C, SK-MEL-131 extract plus TPA; D, AO_2V_4 astrocytoma extract, and D, AO_2V_4 astrocytoma extract plus TPA; E, WI-38 fibroblast extract, and E, WI-38 fibroblast extract plus TPA. Amounts were TPA, 10 ng/ml; cholera toxin, $10^{-8}M$; and cell extracts diluted 1:1000. An epidermal cell suspension derived from a human foreskin (1290) was seeded into tissue culture cluster dishes (12 wells with 2×10^5 cells per well) in cMEM and the factors listed above. Twenty-four hours later, all unattached cells were removed, and each well was refed with the appropriate medium. Three days later the cells were trypsinized and seeded at approximately 1×10^4 cells per well into tissue culture cluster dishes (six wells). Melanocytes were allowed to attach, and later geneticin (100 μg/ml) was added for an overnight treatment (*3*). After treatment, cells were grown for 20 days, trypsinized, and counted. Contamination by fibroblasts was excluded by staining for leucine aminopeptidase–positive cells.

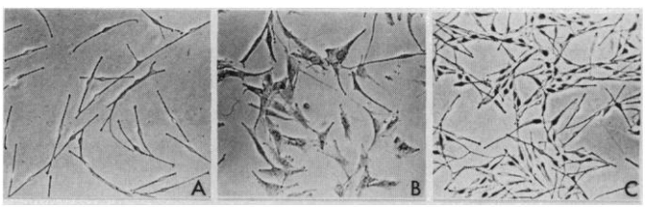

Fig. 3. Morphology of melanocytes cultured in medium containing TPA or melanocyte growth factors derived from the astrocytoma cell line AO_2V_4. (A) TPA, 10 ng/ml; (B) AO_2V_4 astrocytoma extract diluted 1:1000; (C) TPA at 10 ng/ml plus AO_2V_4 astrocytoma extract diluted 1:1000 (magnification, ×200).

bined with cholera toxin was added. Factors in the extracts appear therefore to support the growth of individual melanocytes very efficiently and permit their cloning; this cannot be achieved with cultures grown in TPA or TPA and cholera toxin.

Since known growth factors lacked melanocyte growth-promoting activity, we assume that we are dealing with one or a family of new growth factors. Cell extracts appear to be a far richer source of these new factors than culture supernatants, and it will be important to search for growth factors in extracts of other differentiated cell types. Although it is possible that combinations of known growth factors would reproduce the effects observed with extracts, experiments to test this have yielded negative results. As melanomas and astrocytomas originate from neuroectodermal progenitor cells, production of melanocyte growth factors could be a property of selected cell types derived from this lineage. Support for this idea comes from the recent finding that bovine brain extracts contain a factor capable of stimulating melanocyte growth (*7*). With regard to the potent mitogenic effect of WI-38, fibroblasts are known to have both mesodermal and neuroectodermal origin. In addition, the fact that growth-promoting activity could be extracted from skin fibroblasts raises the possibility that dermal fibroblasts participate in the regulation of melanocytes, an idea that has been widely held. A broader range of normal and malignant cells are being tested to determine the relation of cell type to factor production. Defining the nature and number of these melanocyte growth factors represents the next step in the analysis. The factor from WI-38 appears to be acid- and alkaline-stable, heat-labile, and to have a molecular weight of 40,000 by gel filtration; these characteristics distinguish it from known growth factors.

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Hallucinogenic Amphetamine Selectively Destroys Brain Serotonin Nerve Terminals

Abstract. *(±)-3,4-Methylenedioxyamphetamine (MDA), an amphetamine analog with hallucinogenic activity, produced selective long-lasting reductions in the level of serotonin, the number of serotonin uptake sites, and the concentration of 5-hydroxyindoleacetic acid in rat brain. Morphological studies suggested that these neurochemical deficits were due to serotonin nerve terminal degeneration. These results show that MDA has toxic activity for serotonin neurons in rats and raise the question of whether exposure to MDA and related hallucinogenic amphetamines can produce serotonin neurotoxicity in the human brain.*

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(±)-3,4-Methylenedioxyamphetamine (MDA) is a synthetic amphetamine derivative that produces a mixture of psychomotor stimulatory and hallucinogenic effects (*1*). This combination of psychotropic actions may stem from MDA's

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