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Induction of Megakaryocytic Colony-Stimulating Activity in Mouse Skin by Inflammatory Agents and Tumor Promoters (42474)

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Abstract. The production of megakaryocytic colony-stimulating activity (MEG-CSA) was assayed in acetic acid extracts of skin from mice topically treated with inflammatory and tumor-promoting agents. A rapid induction of MEG-CSA was found in skin treated both with phorbol 12-myristate 13-acetate (PMA), a strong tumor promoter, and with mezerein, a weak tumor promoter, but no induction was found in untreated skin. The time course of induction of MEG-CSA following treatment of skin with PMA or mezerein was very similar to that previously demonstrated for the induction of granulocyte–macrophage colony-stimulating activity in mouse skin by these agents. The induced MEG-CSA was found in both the epidermis and the dermis. Pretreatment of the skin with β-methasone abrogated the MEG-CSA induction. The cell number response curve suggests that the MEG-CSA acts directly on the progenitor cells of the megakaryocyte colonies. That topical administration of diterpene esters results in the rapid, local induction of MEG-CSA which can be blocked by β-methasone pretreatment suggests a mechanism for the thrombocytosis associated with some inflammatory states. The indirect action in which diterpene esters induce in certain cells the production or release of growth regulatory factors for other cell types may also aid in understanding their carcinogenic properties.

Blood cells are intimately involved in the inflammatory response. Infiltration of a tissue by leukocytes is one of the initial events following an inflammatory stimulus, and products released by these cells mediate much of the subsequent response. The skin of mice topically treated with inflammatory agents has been used to study the role of blood cells in the inflammatory process. Mouse skin has also been used to investigate carcinogenesis in the classical two-stage model in which malignant tumors are induced in mouse skin by sequential, topical treatment with initiating and promoting agents. Since an early inflammatory response occurs in skin treated with tumor promoting diterpene esters (1), many investigations have centered on the humoral regulation of myelopoiesis following application of diterpene esters. These agents can induce differentiation and proliferation of both leukemic (2–6) and normal (7–10) myeloid cells. There is evidence for both a direct action of these compounds on myeloid progenitor cells (8) and for an indirect action in which they increase the production of a myeloid cell growth factor by a subpopulation of cells in normal bone marrow (6, 10–12). One of us (M.J.K.) has shown that topical treatment of mouse skin with diterpene esters rapidly induces the production of granulocyte–macrophage colony-stimulating activity (GM-CSA) in skin cells (13). Because thrombocytosis is frequently associated with inflammation and with malignancy, we have used the mouse skin model system to investigate the effects of diterpene esters on growth factors regulating megakaryocytopoiesis. In the present report we show that topically administered inflammatory and tumor-promoting agents rapidly induce megakaryocytic colony-stimulating activity (MEG-CSA) in mouse skin.

Materials and Methods. The diterpene esters, phorbol 12-myristate 13-acetate (PMA) and mezerein were obtained from LC Services Corp. (Woburn, MA). Stock solutions of 10^{-2} *M* in acetone or in dimethylsulfoxide were stored at −80°C. BALB/c and C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Protein concentrations were determined by the Coomassie blue binding method (14).

Skin extracts. For the determination of MEG-CSA in skin, the hair from a 2.0 × 2.5-cm area of the backs of 7- to 10-week-old

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BALB/c mice was shaved 3 days prior to the topical administration of 20 nmol PMA or 20 nmol mezerein each in 0.2 ml acetone. Control mice were treated with 0.2 ml acetone. At various times after treatment, four to seven mice in each treatment group were sacrificed. The treated areas of skin were excised and placed in 0.5% acetic acid in distilled water for 16 hr at 4°C. In some experiments with PMA-treated skin, the epidermis was separated from the dermis following the application of a depilatory cream just prior to sacrifice (13). In these experiments, the abdominal skins of the mice whose backs were treated with PMA were used as controls. The abdominal skins also were treated with depilatory cream, but skin layers were not separated. In some experiments mice were pretreated with 0.025% β-methasone cream (Parke–Davis, Morris Plains, NJ) at 2 hr prior to application of PMA. The acetic acid solutions were dialyzed at 4°C for 48 hr against four changes of distilled water. After determination of their protein content, these extracts were diluted with an equal volume of double strength Iscove's medium, and stored at −20°C. The skin extracts were assayed at a final concentration of 400 μg protein ml in the megakaryocyte colony-forming assay described below.

MEG-CSA assay. Megakaryocytic colony formation was assayed using single cell suspensions of bone marrow from two or three 8- to 12-week-old C57BL/6 mice. Triplicate or quadruplicate aliquots containing 2×10^5 nucleated bone marrow cells were cultured for 5 days in plasma clot cultures at 37°C in a humidified 5% CO_2 atmosphere (15). The cultures consisted of 30% Iscove's medium, 30% α-minimum essential medium, 20% fetal bovine serum, 1% bovine serum albumin, β-mercaptoethanol (10^{-4} *M*) ε-aminocaproic acid (10^{-6} *M*), 120 μg/ml human fibrinogen (Kabi, Uppsala, Sweden), penicillin (200 units/ml), and streptomycin (20 μg/ml). Clotting was initiated by the addition of 10% bovine citrated plasma, and 0.2 units/ml bovine thrombin. After culture, clots were harvested, dehydrated, and fixed to slides with glutaraldehyde. They were stained for acetylcholinesterase activity with a modified Karnovsky–Root reagent and counterstained with hematoxylin. Clusters of two or more positive staining cells were enumerated as colonies.

For comparison with the effects of extracts of treated skin, medium conditioned by pokeweed mitogen-stimulated spleen cells (PWCM) served as a source of MEG-CSA. To produce PWCM, 2.5 μg/ml pokeweed mitogen (Sigma Chemical Co., St. Louis, MO) was added to a suspension of $1–2 \times 10^6$ mouse spleen cells/ml Iscove's medium. The medium was supplemented with antibiotics, 10% heat-inactivated fetal bovine serum, and 1% bovine serum albumin. The supernatant medium was collected after 6 days of incubation.

Results. *Induction of MEG-CSA in mouse skin.* An activity that supported the formation of megakaryocytic colonies was extracted into dilute acetic acid from skins of BALB/c mice that had been treated topically with either PMA or mezerein. Skins treated only with the acetone vehicle did not elaborate this activity (Table I). For comparison with the skin MEG-CSA activities shown in Table I, PWCM supported formation of 186 ± 6 colonies per 2×10^5 cells, when added at a saturating concentration. Increased MEG-CSA was found as early as 3 hr and was maintained for more than 24 hr post PMA or mezerein treatment (Fig. 1). Peak numbers of colonies were about eight times control levels. By 48 hr post treatment the total MEG-CSA from the treated skins approached control levels.

It has been reported that although PMA does not act as a MEG-CSA itself, it can cooperate with other factors to potentiate megakaryocytic colony formation (16). When PMA

TABLE I. EFFECT OF SKIN EXTRACTS ON MEGAKARYOCYTIC COLONY FORMATION

Assayed material	Megakaryocytic colonies/2×10^5 marrow cells[a]
Iscove's medium	4 ± 4
Acetone-treated skin extract	6 ± 5[c]
PMA-treated skin extract[b]	43 ± 8[d]
Mezerein-treated skin extract	48 ± 8[d]

[a] Mean ± SEM of five experiments. Skin extracts were added to cultures at a final concentration of 400 mg protein/ml.

[b] PMA added to marrow cells at concentrations of 3×10^{-12} to 3×10^{-6} *M* produced fewer than four colonies per 2×10^5 cells.

[c] Not significantly different from control.

[d] $P < 0.01$, *t* test.

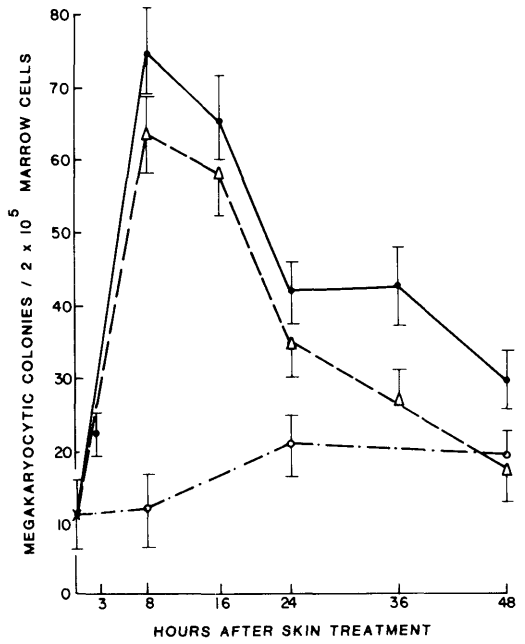

FIG. 1. Time course of induction of MEG-CSA. Megakaryocytic colonies were grown *in vitro* from mouse bone marrow cells in the presence of 400 μg/ml of protein from acetic acid extracts of mouse skin at various times after treatment with PMA (closed circles), mezerein (triangles), or acetone vehicle (open circles). The data point on the ordinate represents baseline colony formation in the absence of addition of skin extract. Each data point is the mean ± SEM of quadruplicate cultures in a representative experiment.

is applied topically as in our experiments, there is a rapid loss of the compound from the skin such that less than 20% is present after 4 hr and less than 10% of the applied dose is present after 12 hr. Since most of the residual PMA is lipid bound and less than 1% of the residue is available for acetic acid extraction (13), concentrations of the order of 10^{-8} *M* could have been expected in the skin extracts (i.e., 10^{-9} *M* in the cultures). We therefore added various concentrations of PMA (3×10^{-12} to 3×10^{-8} *M*) directly to plasma clot cultures. At these concentrations PMA supported no colony formation above the baseline.

In order to identify possible sources of MEG-CSA in the skin, acetic acid extraction was carried out on separated epidermis and dermis from skin of BALB/c mice treated for 24 hr with PMA. The data in Table II show that both the dermis and epidermis of treated skin contained MEG-CSA while the untreated abdominal skin from the same mice showed no induction. Pretreatment of skin with β-methasone largely abolished induction of MEG-CSA by PMA treatment (Table II).

Characteristics of the MEG-CSA. The colony formation in response to MEG-CSA in extracts of PMA treated skin was dose dependent with a plateau in the effect of about 400 μg of extract protein/ml culture medium (Fig. 2). To address the question of whether the MEG-CSA in these extracts acts directly on the megakaryocytic progenitor (CFU-M) or indirectly through accessory cells, cell number-response experiments were conducted (Figure 3). The formation of colonies was linear with respect to cell number from 0.5 to 2.5×10^5 cells per ml of culture suggesting a direct effect of the MEG-CSA on the megakaryocyte colony forming unit (CFU-MEG).

Discussion. The diterpene ester tumor promoters produce an inflammatory reaction in

TABLE II. CHARACTERIZATION OF TISSUES PRODUCING MEG-CSA

Treatment	Tissue extracted[a]	Megakaryocytic colonies/2 × 10^5 marrow cells[b]
Experiment 1		
—	Iscove's medium	3 ± 1
acetone	Whole skin	2 ± 1[c]
PMA	Whole skin	23 ± 3[d]
Experiment 2		
—	Iscove's medium	6 ± 1
None	Abdominal skin[e]	8 ± 1[c]
PMA	Epidermis	27 ± 3[d]
PMA	Dermis	20 ± 3[d]
Experiment 3		
—	Iscove's medium	6 ± 1
PMA	Whole skin	33 ± 5[f]
β-methasone + PMA	Whole skin	7 ± 1[c]

[a] Tissue extracts were added to cultures at a final protein concentration of 400 μg/ml. Iscove's medium was added in place of extract to determine baseline colony formation.

[b] Mean ± SEM of quadruplicate or triplicate determinations in representative experiments.

[c] Not significantly different from control.

[d] $P < 0.001$, *t* test.

[e] Whole abdominal skin from mice whose back skins were treated with PMA. Both abdominal and back skins were treated with depilatory cream immediately prior to sacrifice.

[f] $P < 0.01$, *t* test.

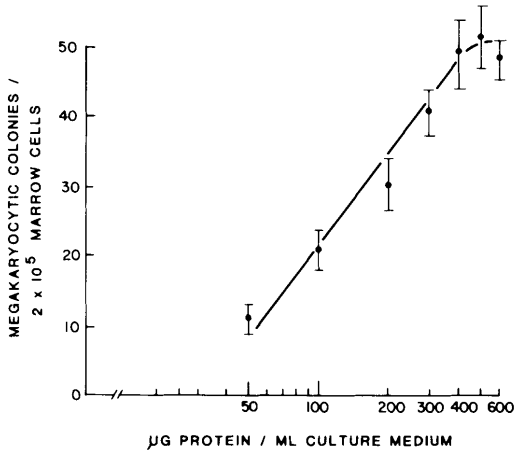

FIG. 2. Dose-response curve of megakaryocytic colony number versus PMA-treated skin extract added to cultures. The amount of skin extract is quantitated as micrograms of total protein in the final culture medium, and the colonies are quantitated as number per 2×10^5 bone marrow cells cultured. Each data point represents the mean ± SEM of two separate experiments.

skin and have marked effects on the proliferation and differentiation of various cell types *in vivo* and *in vitro*. Our results show that these agents can exert a stimulatory effect on the proliferation of progenitors of megakaryocytes through the induction of MEG-CSA. Both the strong tumor promoter, PMA, and the weak tumor promoter, mezerein, induced this activity, and this induction was largely abolished by pretreatment with an anti-inflammatory steroid. Thus, MEG-CSA induction appears to correlate with the inflammatory reaction itself, rather than with the specific tumor-promoting ability of these compounds. The cells in the skin which responded to the diterpene esters do not appear to be confined to lymphocytes or other leukocytes. Within the skin both dermis and epidermis contained acetic acid-extractable MEG-CSA. In histologic studies, leukocytic (almost exclusively polymorphonuclear) infiltration of the skin is very intense by 24 hr after treatment with PMA, but it is confined to the dermis (13). The results of the separation experiments demonstrate that MEG-CSA production occurs both in the dermis, which is infiltrated with leukocytes, and in the epidermis, which is not. These results cannot rule out completely the possibility that the epidermal activity is derived by diffusion from the dermis or vice versa. The finding that epidermal cells can produce hematopoietic growth factors has previously been demonstrated. Sauder *et al.* (17) showed that stimulation of cultured mouse epidermal keratinocytes by PMA leads to the elaboration of an epidermal cell thymocyte-activating factor, and Koury *et al.* (13) reported the production of GM-CSA in the skin of mice treated with diterpene esters.

At present the MEG-CSA induced in mouse skin by the diterpene esters is not characterized completely. The cell number response curve suggests that the activity is due to a factor which acts directly on the CFU-M. In addition to a putatively specific megakaryocytic CSF (18) a number of other hematopoietic growth factors have been shown to support megakaryocytic colony formation in mouse bone marrow cultured in the presence of serum. These include interleukin 3 (IL-3) (19), GM-CSA-2 (19), and erythropoietin (20, 21). Neither IL-3 nor erythropoietin appears to be responsible for the MEG-CSA we have demonstrated. No erythroid cell growth could be

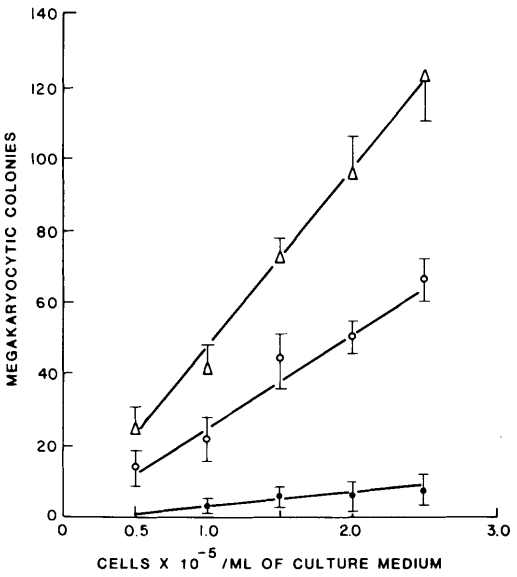

FIG. 3. Cell number response curve. The number of megakaryocytic colonies grown is plotted versus the cell concentration with three different potential growth stimulators: PWCM, 10% final concentration (triangles), extract of PMA-treated skin, 400 μg/ml final extract protein concentration (open circles), or extract of acetone-treated skin, 400 μg/ml final extract protein concentration (closed circles). Each data point represents the mean ± SEM of quadruplicate cultures in a representative experiment.

found in any cultures. Although GM-CSA messenger RNA is present in the epidermis of PMA-treated mouse skin, no messenger RNA for IL-3 can be detected in PMA-treated skin (I. B. Pragnell, personal communication). On the other hand, the time course of induction of MEG-CSA is nearly identical to that demonstrated for GM-CSA (13), and the GM-CSA also is blocked by pretreatment of the skin with β-methasone (unpublished results). Further work is required to ascertain the molecular nature of the induced MEG-CSA and its relationship to GM-CSA.

The induction in non-lymphoid–hematopoietic tissues of factors that stimulate the proliferation of blood cells may help to explain the leukocytosis and thrombocytosis frequently observed in inflammation. And it is possible that the elaboration of growth factors capable of stimulating the proliferation of a variety of cell types during the course of the inflammatory response may be involved in the link between inflammation and carcinogenesis.

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