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Induction of granulocyte-macrophage colony-stimulating activity in mouse skin by inflammatory agents and tumor promoters

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The granulocyte-macrophage colony stimulating activity (GM-CSA) was assayed in acetic acid extracts of skin from mice which were topically treated with inflammatory and tumor-promoting diterpene esters. Extremely large increases in GM-CSA were found in skin treated with the strongly tumor-promoting 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and the weakly promoting mezerein, while only a very slight increase was found with the non-promoting 4-O-methyl-TPA (4-OMe-TPA). Untreated areas of skin had very little GM-CSA. In the treated skins, the elevated GM-CSA was noted within a few hours and lasted for > 24 h after treatment. Although the levels of GM-CSA induced in the skin correspond to the degree of inflammation elicited by the respective treatments, the leukocytes in the acute inflammatory infiltrate did not appear to be responsible for the increased GM-CSA. Both epidermis and dermis had increased GM-CSA following TPA treatment of skin. Treatment of fibroblast and epithelial continuous cell lines with diterpene esters resulted in a similar pattern of GM-CSA induction in their supernatant media as that noted in the skin extracts. A large majority of the colonies stimulated by the diterpene-ester induced GM-CSA were composed of only macrophages. The results demonstrate that the topical administration of an inflammatory diterpene ester results in a rapid, marked yet local GM-CSA induction in the skin of treated mice. This indirect action in which diterpene esters induce in certain cells a growth regulatory factor for other types of cells may be an important element in carcinogenesis.

Key words: GM-CSA/growth factors/inflammation/skin carcinogenesis/tumor promoters

Introduction

In the classic two stage model of carcinogenesis (Berenblum, 1941), malignant tumors are induced in mouse skin by sequential, topical treatment with initiating and promoting agents. The validity of this model system has been extended to a wide variety of tissues and cell types (Sivak, 1979). Among the various responses occurring within hours of tumor promoter treatment in mouse skin are the inflammatory changes of vasodilatation, edema, and leukocytic infiltration (Frei and Stephen, 1968). These effects, which are observed mainly in the dermis, precede a proliferation of epidermal cells which becomes evident by 24 h after treatment. Many investigations have been concerned with the identification of specific proteins induced in mouse skin during the inflammatory period which may affect the subsequent epidermal proliferation and tumor promotion. Specific proteins and glycoproteins induced or greatly increased in mouse skin within hours of diterpene ester treatment have been identified by polyacrylamide gel electrophoresis (Scribner and Boutwell, 1972; Balmain, 1976; Gottesman and Yuspa, 1981). The source of these proteins appears to be the epidermis, but their biological functions have not been determined. The enzyme activities of polyamine synthesis (O'Brien *et al.*, 1977) and protein kinase (Murray and Froscio, 1977) in the epidermis and of histidine decarboxylase (Taguchi *et al.*. 1982) in the dermis are increased within hours of tumor promoter treatment, but the relationships of these activities to cellular proliferation and subsequent tumor promotion are not established.

The early inflammatory response in mouse skin appears to be a common reaction to treatment with all of the tumor-promoting diterpene esters (Hecker, 1978). Although the inflammation may be necessary, it is not sufficient for tumor promotion since some diterpene esters such as mezerein (Mufson *et al.*, 1979) or phorbol-12-retinoate-13-acetate (Furstenberger *et al.*, 1981) elicit inflammation, but have little tumor-promoting ability. Among the cells involved in inflammatory responses, the blood leukocytes have been the subject of numerous studies *in vitro* with tumor-promoting diterpene esters. In particular, the diterpene esters have been shown to induce differentiation and proliferation of leukemic myeloid cells (Rovera *et al.*, 1979; Huberman and Callahan, 1979; Pegaroro *et al.*, 1980; Koeffler, 1981; Lotem and Sachs, 1979) and myeloid progenitor cells from normal bone marrow (Lotem and Sachs, 1979; Stuart *et al.*, 1981; Fibach *et al.*, 1980; Abrahm and Smiley, 1981; Greenberger *et al.*, 1980a). In these latter studies, evidence has been presented for direct action of the diterpene esters on the myeloid progenitor cells (Stuart *et al.*, 1981). Other evidence has been presented for an indirect action of these agents in that they increase the production of a myeloid progenitor growth factor by a subpopulation of cells in the normal marrow. Specifically, the diterpene esters have been shown to increase the production of granulocyte-macrophage colony stimulating activity (GM-CSA) by cells with a mature monocytic-macrophagic phenotype (Lotem and Sachs, 1979; Greenberger *et al.*, 1980a; Svet-Moldavskaya *et al.*, 1981; Ralph *et al.*, 1977). GM-CSA, a glycoprotein(s) produced by a wide variety of cells, induces the progenitors of granulocyte and macrophage cells to proliferate and differentiate *in vitro* to form discrete colonies of granulocytes and/or macrophages (Burgess and Metcalf, 1980).

In view of the reports that GM-CSA could be induced by diterpene esters in mature monocyte-macrophage cells and since other malignancy-associated agents, retroviruses, have been shown to induce GM-CSA in macrophages (Greenberger *et al.*, 1980b) and fibroblasts (Koury and Pragnell, 1982), we decided to investigate the induction of GM-CSA in the skin of mice treated topically with diterpene esters. Our results demonstrate that tumor-promoting doses of the diterpene esters rapidly induce GM-CSA production in skin. This increased GM-CSA activity, which is detected in both the dermis and epidermis, stimulates growth of predominantly macrophage colonies. The induction appears to be

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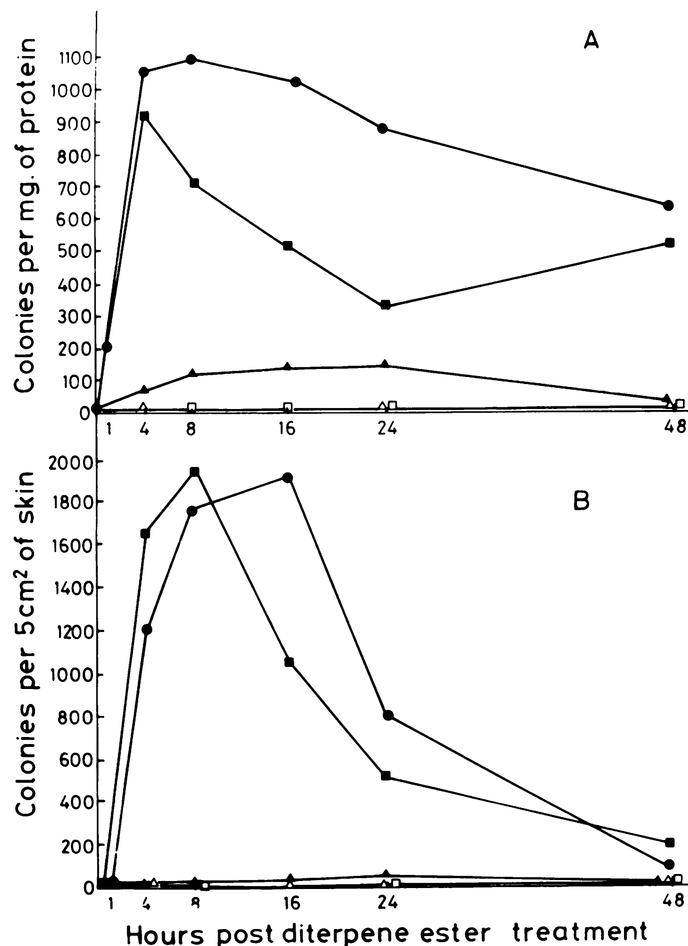

Fig. 1. Number of granulocyte-macrophage colonies stimulated by acetic acid extracts of mouse skin at various times following diterpene ester treatment. (**A**) Colonies per mg of extracted protein, (**B**) colonies per 5 cm² of skin. The data represent the average of two experiments using marrow cells from BALB/c mice. Similar results were found with NIH-Swiss marrow cells. Treatments were: (●) TPA 20 nmol, (■) mezerein 20 nmol, (▲) 4-OMe-TPA 400 nmol, (□) acetone 0.2 ml, (△) untreated abdominal skin from mice treated on the back with TPA.

closely associated with ability of the specific diterpene ester to elicit an inflammatory response. These results demonstrate that the induction of a growth factor for myeloid cell progenitors is an early response of normal skin to treatment with diterpene esters.

Results

Induction of GM-CSA in mouse skin

Mice were topically treated on their skin with the strongly tumor-promoting 12-O-tetradecanoyl-phorbol-13-acetate (TPA), the weakly promoting mezerein or the non-promoting 4-O-methyl TPA (4-OMe-TPA). Increased GM-CSA was found as early as 1 h and was maintained for more than 24 h post-TPA or mezerein treatment (Figure 1). Peak numbers of colonies at 8–16 h post-treatment were > 1000 times control levels. Since variable amounts of edema in the skins of mice treated with TPA and mezerein were noted at 4–24 h post-treatment, the results in Figure 1 are expressed in GM-CSA per mg of extracted protein (A) and per unit area of skin (B). The increase in GM-CSA, which was detected within a few hours of treatment with diterpene esters, was much greater with TPA and mezerein than with 4-OMe-TPA (Figure 1). By 48 h post-treatment, the total GM-CSA from the treated skins approached control levels. Skins treated with acetone had very little GM-CSA. Untreated skins from the abdomen of TPA-treated mice also showed very little GM-CSA, indicating that the GM-CSA induction by TPA occurs only in the treated area of skin.

Since some previous reports showed a synergistic colony-forming activity between GM-CSA and TPA (Lotem and Sachs, 1979; Stuart *et al.*, 1981; Fibach *et al.*, 1980), experiments were designed to determine whether such a synergism could account for the observed GM-CSA in treated skins. Experiments with tritium-labeled TPA have shown that, when it is administered topically as in our experiments, there is a rapid loss, such that in the treated area of skin < 20% is present after 4 h and < 10% of the applied dose is present after 12 h (Kriebich *et al.*, 1971). Since the vast majority of this residual TPA is lipid-bound and only 1% or less of this residue is available for extraction in an aqueous solution of acetic acid (Helmes *et al.*, 1974), concentrations of the order of 10^{-8} could have been expected in the solutions of skin extracts (i.e., ~ 10^{-9} M in the GM-CSA assay) when they were redissolved at 1.0 mg protein/ml. When such concentrations of TPA were added to the extracts of normal, untreated skin there was no evidence of a synergistic increase in colony formation between the TPA and the low levels of GM-CSA in the normal skin. Thus, *de novo* synthesis accounted for the observed increase in skin GM-CSA. The only possible exception was the 1 h post-TPA skin where the residual TPA was still at relatively high levels and the protein extracted per unit area of skin was small. In this case the redissolved protein solution could contain TPA at levels of 10^{-7} M or greater. The average of ~200 colonies per mg protein in the 1 h sample was between the 160 colonies per mg protein found when the normal skin extract was made to 10^{-7} M with added TPA and 300 colonies per mg protein when it was made to 10^{-6} M TPA. Thus, synergism between residual TPA and the low constitutive GM-CSA levels in the skin may have accounted for the GM-CSA activity being increased at such an early time as 1 h after treatment, but it did not account for the very large increases seen at later times.

Relation of skin cell types to GM-CSA production

Since previous reports indicated that mature macrophages may be a source of diterpene ester-induced GM-CSA and since an influx of inflammatory cells occurs soon after diterpene ester treatment of skin, histological sections of treated skins were examined to determine when the infiltration occurred relative to the GM-CSA increase as well as which cell types were involved in the infiltration. As TPA and mezerein have been shown to have the same effects on dermal inflammation and epidermal proliferation (Mufson *et al.*, 1979) only skins treated with TPA and 4-OMe-TPA were examined histologically. At 1 h after treatment with TPA the skin was histologically similar to control skin (Figure 2A). By 4 h post-treatment when GM-CSF activity is high, edema and vasodilatation in the dermis were prominent but no leukocytic infiltrate was present (Figure 2B). Dermal leukocytic infiltration, first observed at 8 h post-treatment, reached a peak at 16–24 h, and largely subsided by 48 h (Figure 2C and D). At all times the vast majority of infiltrating cells were polymorphonuclear luekocytes confirming previous reported results (Frei and Stephen, 1968). Although the skins treated with 4-OMe-TPA showed active mitosis in the basal layer and

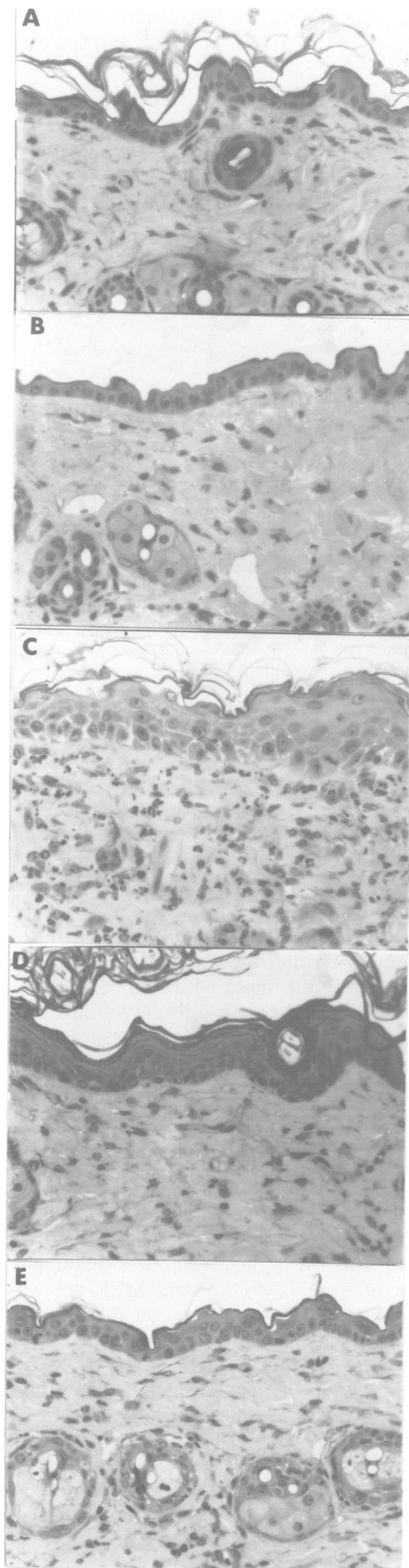

Table I. GM-CSA in epidermis and dermis following topical TPA treatment of mouse skin

Hours post TPA	Skin fraction	Colonies/ mg protein	Colonies/ 5 cm² skin	Percent macrophage colonies
4	Epidermis	1020	1096	88
	Dermis	260	2184	83
24	Epidermis	730	688	94
	Dermis	360	1296	89

Acetic acid extracts of the separated skin fractions were redissolved to 1.0 mg/ml and assayed at 20% of final culture volume for GM-CSA as described in Materials and methods.

some epithelial proliferation at 24 h post-treatment (Figure 2E), extremely little leukocytic infiltrate was found at any time during the 48 h period following treatment.

In order to identify more accurately the source(s) of the GM-CSA in the skin, acetic acid extraction was carried out on separated epidermis and dermis from skin treated for 4 and 24 h with TPA (Table I). The specific activity is greater in the epidermis than the dermis but the total amount of GM-CSA activity was greater in the dermis at both times. These findings are most probably due to the large amounts of edema-related protein in the dermis. The results of these separation experiments together with the histological studies demonstrate GM-CSA production at 4 and 24 h both by epidermis which is not infiltrated with leukocytes at any time and by dermis which is not infiltrated at 4 h but is at 24 h post-TPA treatment. These results cannot completely rule out the rather unlikely possibility that the activity in the epidermis is derived by diffusion from the dermis.

Induction of GM-CSF in vitro in fibroblast and epithelial cells

To confirm that cells other than resident or infiltrating leukocytes were the sources of GM-CSA in the treated skin, we examined the effects of diterpene esters *in vitro* on epithelial (MMCE) and fibroblast (FRE) continuous cell lines. The two cell lines were selected because their supernatant media have extremely little constitutive production of GM-CSA. Figure 3 shows the increased GM-CSA activity found in the supernatant media of these cell lines following exposure to diterpene esters. The general pattern of greater GM-CSA induction with TPA and mezerein than with 4-OMe-TPA is similar to that noted in the skin. Although GM-CSA activity is increased in these cell line supernatants following treatment, it is not certain how much of the activity in the supernatants is due to a large increase in GM-CSA or due to a synergistic effect between small increases in GM-CSA and the relatively high concentrations of TPA in the medium. To test these two possibilities, reconstitution experiments were done in which TPA was added to the GM-CSA assay of supernatants from untreated cells. In these experiments no significant increase was seen above the GM-CSA found when TPA was added alone to the assays (Figure 3).

Fig. 2. Histological sections from skins following treatment with diterpene esters as described in Materials and methods. **(A)** Normal skin, **(B)** 4 h post-TPA, **(C)** 24 h post-TPA, **(D)** 48 h post-TPA, **(E)** 24 h post-4-OMe-TPA. Hematoxylin and eosin stained x 210.

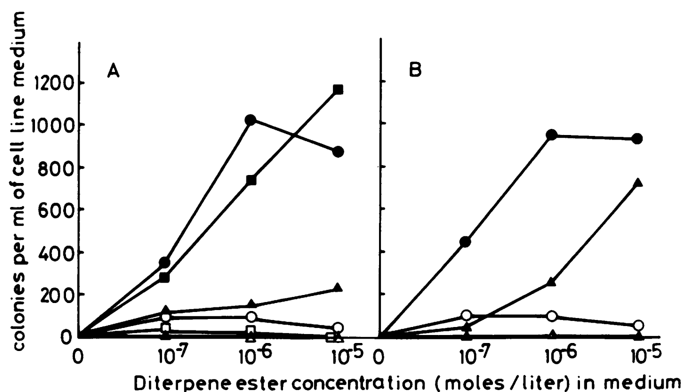

Fig. 3. Number of granulocyte-macrophage colonies stimulated by supernatant media of continuous cell lines cultured in the presence of TPA. (A) FRE cells, (B) MMCE cells, (●) medium of TPA-treated cell culture, (○) TPA medium control, not incubated with cells; (■) mezerein cell medium, (□) mezerein control medium; (▲) 4-OMe-TPA cell medium, (△) 4-OMe-TPA control medium. TPA concentrations are those for cell line medium (i.e., only 20% of these concentrations are present in GM-CSA assays).

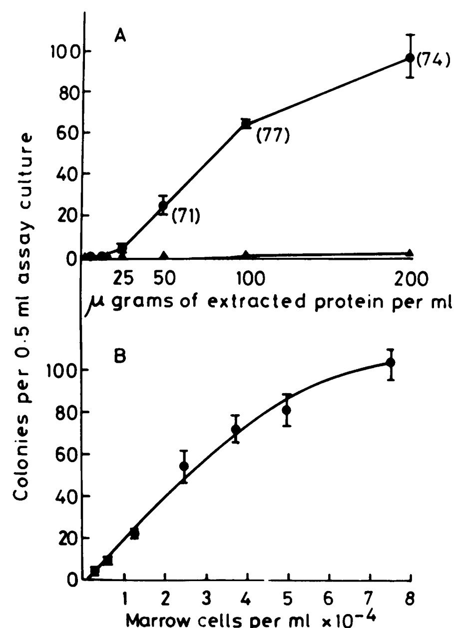

Fig. 4. (A) Number of granulocyte-macrophage colonies stimulated *versus* concentration of acetic acid extract in assay culture. (●) 16 h post-TPA, (▲) 16 h post-acetone. Numbers in parenthesis show percentages of pure macrophage colonies. Data are ± 1 S.D. for triplicate 0.5 ml cultures of 7.5 x 10^4 marrow cells/ml. (B) Number of granulocyte-macrophage colonies stimulated by 16 h post-TPA extract *versus* number bone marrow cells in assay culture. Data are ± 1 S.D. for triplicate 0.5 ml cultures.

Thus, most of the induced activity is attributable to *de novo* production of GM-CSA by the treated cells. A macrophage colony predominance similar to that found with the skin extracts (see below) was found in the assays of supernatant media.

Characteristics of GM-CSA induced in skin

The colonies stimulated by the skin extracts were 74–93% pure macrophage type with TPA treatment, 79–91% with mezerein and 90–97% with 4-OMe-TPA. Of the remaining 5–20% of colonies, about two-thirds were mixed macrophage-granulocyte type and one-third were pure granulocyte type. An association between diterpene esters and increased proportions of macrophage colonies has been noted in several other studies (Fibach *et al.*, 1980; Abrahm and Smiley, 1981; Greenberger *et al.*, 1980a). We also have found that 10^{-7}–10^{-9} M TPA or mezerein when included in the colony-forming assay with a GM-CSA supernatant medium from SC1 cell line (Koury and Pragnell, 1982) which normally stimulates 50% or less macrophage colonies will stimulate increased numbers of colonies of which 80% or more are pure macrophage colonies. Thus, although the induction in skin of a GM-CSA which stimulates mainly pure macrophage colony progenitors is a possibility, the diterpene esters may also have an effect directing the differentiation of the granulocyte-macrophage progenitor along the macrophage pathway which is distinct from their indirect, GM-CSA mediated effect on the progenitor's proliferation.

The GM-CSA was stable after heating at 56°C for 30 min. Figure 4A shows a dose-response relationship between the extracted GM-CSA in TPA-treated skin and colony number and percentage of pure macrophage type. The linear slope which approaches zero as cells are decreased in the GM-CSA (Figure 4B) suggests that the GM-CSA in the skin acts directly on the colony forming progenitor cell. A purified preparation of major excreted protein (MEP), a glycoprotein secreted after TPA treatment of fibroblasts and known to be rapidly induced in TPA-treated mouse skin (Gottesman and Yuspa, 1981), did not have any GM-CSA.

Discussion

The diterpene ester tumor promoters have marked effects on the proliferation and differentiation of various cell types both *in vivo* and *in vitro*. Our results show that these tumor-promoting agents can exert their proliferation and differentiation effects on progenitors of macrophages and granulocytes indirectly through the induction of GM-CSA in non-hematopoietic cells. A similar indirect stimulation of macrophage-granulocyte progenitor cells via fibroblast production of GM-CSA has been found with retroviruses which induce proliferation of hematopoietic cells *in vivo* and are associated with the generation of leukemia. In this respect it is interesting that an increased incidence of leukemia has been found in carcinogen-initiated mice which were treated topically with tumor-promoting diterpene esters (Goerttler and Loehrke, 1977).

The glycoprotein MEP has been found to be strongly induced by retrovirus (Kirsten sarcoma virus), TPA and mezerein but only very slightly induced by 4-OMe-TPA (Gottesman and Yuspa, 1981; Gottesman and Sobel, 1980). In mouse skin treated with diterpene esters the time courses for production of both GM-CSA and MEP are very similar. These numerous similarities between GM-CSA and MEP suggested that they may be related. A purified preparation of MEP, however, had no GM-CSA in our assay.

With systemically applied stimuli such as total body irradiation or injection of endotoxin or poly(A)-poly(U), it has been shown that in many different organs GM-CSA is induced within a few hours (Sheridan and Metcalf, 1972). We have shown here that diterpene esters applied topically result in a rapid increase in GM-CSA activity in the treated area of skin but not in areas of skin which were not treated. These findings suggest that when the appropriate stimulus is applied to

an organ or tissue the production of GM-CSA is an early response. Whether the induced GM-CSA acts locally or at a distant site such as the hematopoietic organs is not certain. Despite numerous attempts, we were unable to demonstrate a significant change in serum GM-CSA in these topically treated mice. Since the granulocyte-macrophage colony-forming cells are present in blood (Burgess and Metcalf, 1980) it may be possible that during the inflammatory response following diterpene ester administration these progenitor cells could enter the skin where their proliferation and differentiation would be influenced by locally produced GM-CSA. Experiments designed to test this possibility are currently being undertaken.

Some previous studies showed that the effects of TPA on proliferation and differentiation of normal and leukemic myeloid cells can be due to GM-CSA produced by mature macrophage cells in the treated hematopoietic cell population. Our studies, both *in vivo* and *in vitro*, show that cells which respond to the diterpene esters with increased GM-CSA production are not confined to hematopoietic tissue or mature macrophages, but can be of other types, such as epidermal cells and fibroblasts. Within the skin, both the epidermis and dermis contained acetic acid-extractable GM-CSA. Although the epidermis consists of predominantly one cell type, the dermis consists of many types of cells other than fibroblasts which could contribute to the GM-CSA production. Endothelial cells, macrophages, and lymphocytes are present in normal dermis and each of these cell types has been shown to be able to produce GM-CSA (Burgess and Metcalf, 1980). All of these cells and fibroblasts are involved in inflammatory reactions. The increased GM-CSA in the skin prior to the acute inflammatory infiltration and the preponderance in the infiltrate of polymorphonuclear leukocytes which do not produce GM-CSA suggest that the acutely infiltrating cells do not play a major role in increasing the GM-CSA of skin treated with diterpene esters. This interpretation is also supported by the absence of the acute inflammatory infiltrate in the epidermis which had high GM-CSA.

The finding that epidermal cells can produce a hematopoietic growth factor is not without precedent. Luger *et al.* (1981) have demonstrated the production of epidermal cell thymocyte activating factor (ETAF) by cultures of transformed mouse keratinocytes. This factor, which is similar or identical to murine interleukin 1 (Luger *et al.*, 1982), is also produced by primary cultures of epidermal keratinocytes, particularly after stimulation with TPA (Sauder *et al.*, 1982). It is therefore possible that the production of lymphokine growth factors by epidermal cells may be an important response to tissue damage resulting in inflammation.

The much higher GM-CSA activities induced in skin by TPA and mezerein as compared with 4-OMe-TPA correlate well with the greater amounts of acute inflammation and epidermal proliferation caused in the skin by the former two agents. Mezerein, however, is a very weak tumor promoter when compared with TPA and therefore the induction of GM-CSA correlates better with the inflammatory and epidermal proliferative capabilities of the diterpene ester rather than with its tumor-promoting capability. It is quite possible that GM-CSA is involved in the inflammatory response of skin to diterpene esters because the progeny of GM-CSA target cells – granulocytes and macrophages – play a key role in inflammation. The relationship of the inflammatory response to eventual tumor promotion is unclear. However, experiments with diterpene esters showing marked reduction in tumor promotion following corticosteroid inhibition of the inflammatory response (Belman and Troll, 1972; Viaje *et al.*, 1977), suggest an important relationship *in vivo* between the two events.

Our studies have shown that in a tissue composed of heterogeneous types of cells such as skin, the diterpene esters can act indirectly through the induction in certain cells of a growth regulatory factor for other types of cells. We are currently investigating whether the diterpene esters induce other growth factors which stimulate the proliferation of epidermal and fibroblast cells.

Materials and methods

The diterpene esters TPA, 4-OMe-TPA and mezerein were gifts from G. Furstenberger and E. Hecker (German Cancer Research Centre, Heidelberg). Stock solutions at 10^{-2} M in acetone were stored at $-80°C$. Purified major excreted protein (MEP) was a gift from M. Gottesman (Laboratory of Molecular Biology, NCI). Inbred BALB/c and NIH Swiss mice were obtained from Olac 1976 Ltd., UK. The Fisher rat embryo fibroblast (FRE) and mouse embryo epithelial (MMCE) cell lines as well as their lack of constitutive GM-CSF production have been described (Koury and Pragnell, 1982). Protein concentrations were determined by the Coomassie blue binding method (Bradford, 1976).

For determination of GM-CSA in skin, the hair from a 2.0 x 2.5 cm area on the backs of 7-week-old NIH Swiss mice was shaved at 3 days prior to topical administration of 20 nmol TPA, 20 nmol mezerein or 400 nmol of 4-OMe-TPA each in 0.2 ml of acetone. A previous report had shown that this dose of 4-OMe-TPA, although not tumor-promoting, did stimulate epidermal proliferation (Furstenberger and Marks, 1980). Control mice were treated with 0.2 ml of acetone. At various times after treatment, four to seven mice per treatment group were sacrificed. The treated areas of skin and, for some time points, equivalent areas of shaved, untreated skin over the abdomen were excised. The skins were placed in 0.5% acetic acid in distilled water for 16 h at 0°C as described previously (Balmain, 1976). In some experiments with TPA-treated skin, the epidermis was separated from the dermis after applying a depilatory cream (Balmain, 1976). The acetic acid solutions were dialysed at 4°C for 48 h against four changes of distilled water. After determination of their protein content, these extract solutions were freeze-dried, redissolved in Dulbecco's modified Eagle's minimal essential medium (DMEM) at 1.0 mg protein/ml, and stored at $-20°C$. Skin samples for histological sections, taken just prior to placing the skins in acetic acid, were fixed in 5% formalin.

To examine GM-CSA production *in vitro*, the continuous cell lines FRE and MMCE were incubated in 25 cm^2 area plastic flasks at 37°C in air plus 5% CO_2. The culture medium was DMEM plus 10% fetal calf serum. When the cell growth was subconfluent, the medium was changed and the diterpene esters added. The cells were then cultured for 2 days and supernatant medium was collected. The cells in the flasks were treated with trypsin, counted and discarded. Supernatant media were adjusted to a volume equivalent of 10^6 cells/ml, filtered through 0.2 μm filters, and stored at $-20°C$. Control media containing various concentrations of the diterpene esters, but not in contact with the cell lines, were incubated, filtered and stored in a similar manner.

GM-CSA was assayed using single cell suspensions of bone marrow from 8–10 week old mice. Triplicate 0.5 ml aliquots of 7.5 x 10^4 bone marrow cells/ml were cultured in methylcellulose medium as described previously (Koury and Pragnell, 1982). The protein extracts or supernatant media comprised 20% of the final assay medium. In preliminary experiments, we found that TPA added from $10^{-6}-10^{-9}$ M in the GM-CSA assays stimulated some colonies in the absence of added GM-CSA. The number of colonies formed varied not so much with the TPA concentration but with the fetal calf serum used. All data in the results were obtained with a fetal calf serum which gave the least number of colonies (usually < 10 per 0.5 ml culture) when TPA was added alone to the assays. Colonies of 50 or more cells were scored on the seventh day of culture. Identification of cell phenotypes in the colonies was confirmed with clotted, stained cultures (Koury and Pragnell, 1982).

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