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Inhibition of DNA and Protein Synthesis in UV-Irradiated Mouse Skin by 2-Difluoromethylornithine, Methylglyoxal bis(Guanylhydrazone), and Their Combination

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Exposure of mouse skin to UVB irradiation greatly enhanced the biosynthesis and accumulation of putrescine and spermidine before or concomitantly with stimulation of epidermal macromolecular (DNA and protein) synthesis. Topical treatment of UV-exposed skin with 2 inhibitors of polyamine biosynthesis, 2-difluoromethylornithine (DFMO) and methylglyoxal bis(guanylhydrazone) (MGBG) prevented the enhanced epidermal accumulation of polyamines, especially spermidine, and also inhibited the incorporation of radioactive precursors into DNA and protein. When applied in combination, these 2 antimetabolites of polyamines produced an inhibition of macromolecular synthesis that was at least additive: [^{3}H]thymidine incorporation decreased by 80% and [^{14}C]leucine incorporation by 44% as compared with the UVB-irradiated control mice. A slight decrease in the ratio of [^{3}H]histidine/[^{14}C]leucine incorporation indicated that protein synthesis of the differentiating cell layers was also affected by the inhibitors. The effects of the combined DFMO and MGBG treatment were partially reversed by concomitant topical application of spermidine.

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Abbreviations:
DFMO: 2-difluoromethylornithine
MGBG: methylglyoxal bis(guanylhydrazone)

Interest in polyamine metabolism among dermatologists arose in mid 1970s, when Proctor et al [1] first reported that blood of psoriatic patients contains high concentrations of spermidine and spermine. Since then, elevated polyamine levels have been reported in psoriatic lesions [2–4] and in the urine of psoriatic patients [3]. Moreover, successful treatment of the disease resulted in a decline of the polyamine concentrations approximately to the levels found in healthy epidermis [4]. Enhanced polyamine synthesis was similarly reported in human cutaneous epitheliomas [5].

Presently, experimental data obtained with animal models indicate that epidermal proliferation is invariably accompanied by an enhancement of polyamine biosynthesis; wound healing [6], induction of skin tumors by tumor promoters [7–9], and UVB irradiation [10–12] all result in an increased polyamine accumulation in the affected epidermis. Further, topical application of putrescine or spermine has been reported to stimulate DNA synthesis in mouse epidermis [13].

The synthesis of the physiologic polyamines, is regulated mainly by 2 inducible enzymes, ornithine decarboxylase and adenosylmethionine decarboxylase. Ornithine decarboxylase catalyzes the formation of putrescine from ornithine, while adenosylmethionine decarboxylase produces decarboxylated S-adenosylmethionine which is required for the synthesis of spermidine (from putrescine) and spermine (from spermidine) [14]. In mouse skin, DL-hydrazinoaminovaleric acid, a competitive inhibitor of ornithine decarboxylase inhibits the induction of skin proliferation produced by ethylphenylpropiolate [15]. Tumor formation in mouse skin is almost totally prevented by 2-difluoromethylornithine (DFMO) [16], a highly specific and practically nontoxic irreversible inhibitor of ornithine decarboxylase [17]. However, Seiler and Knödgen [11] obtained only a marginal inhibition of mouse epidermal DNA synthesis by

peroral DFMO treatment. Methylglyoxal bis(guanylhydrazone) (MGBG) is a potent competitive inhibitor of adenosylmethionine decarboxylase [18], which at millimolar concentrations also produces extensive mitochondrial damage [19,20] and causes rapid cell death [21]. In their work with cultured human keratinocytes, Proctor et al [22] showed that micromolar concentrations of MGBG inhibited keratinocyte proliferation and promoted their differentiation. The extent of differentiation was estimated by using the ratio of histidine/leucine incorporation into the cellular protein, which reflects the rate of the synthesis of histidine-rich keratinous proteins occurring preferably in the granular layer.

We have recently shown that polyamine depletion produced by DFMO treatment greatly increases the uptake of MGBG into mouse epidermis [23], a phenomenon previously well documented not only in cell cultures [24] and experimental animals [25], but also in human leukemia [26]. The theoretical basis of this stimulation is based on the fact that polyamine-deprived cells attempt to normalize abnormally low intracellular polyamine pools by enhancing the uptake of extracellular polyamines [27]. As MGBG is structurally a congener of spermidine, it is actively transported into polyamine-deficient cells via the polyamine uptake system [21,28].

Since systemic administration of MGBG is likely to produce undesirable side effects, we developed a convenient topical application system for DFMO and MGBG [29]. In the present work we will show that a topical treatment of mouse skin with DFMO and MGBG, especially in combination, prevents the UVB-induced epidermal polyamine accumulation and results in a profound inhibition of DNA synthesis and, to a lesser extent, protein synthesis. Topical spermidine partially reversed the inhibitory effects of the combined DFMO and MGBG on the macromolecular synthesis.

MATERIALS AND METHODS

Animals and Treatments

Female albino mice were used in all experiments. After an initial haircut with scissors, the dorsal hair was removed with the aid of cream hair remover. Only animals showing no hair regrowth were used.

The animals treated with DFMO received the drug in drinking water (3% solution) for 3 days before the UV irradiation. After the irradiation DFMO was applied topically as a 20% ointment (Ambilan base, Orion, Espoo, Finland) twice a day. MGBG and spermidine were applied as 50 mM ointments also twice a day, starting immediately after the irradiation. When DFMO and MGBG, or DFMO, MGBG, and spermidine were given together, all compounds were combined in a single ointment. The topical treatment was continued for 48 h, thus involving 4 drug applications. The skin was washed 2 h before killing, and the preparation of the epidermal samples was performed as described previously [23]. Each experimental group consisted of 5 mice.

Irradiation of the skin was accomplished with a UVB lamp (λ_{max} 310 nm, Airam, Finland) for 10 min at a distance of 40 cm (total dose 0.15 J/cm). For the determination of radioactive incorporation into DNA and protein, 25 μCi of [^{3}H]thymidine, 2.5 μCi of [^{14}C]leucine, and 5.0 μCi of [^{3}H]histidine was injected i.p. 1 h before killing.

Chemicals

Difluoromethylornithine was a generous gift from the Centre de Recherche Merrell International (Strasbourg, France). The diacetate salt of methylglyoxal bis(guanylhydrazone) was synthesized by Orion Pharmaceutical Company (Espoo, Finland). S-Adenosyl-L-[1-^{14}C]methionine was prepared enzymically as described in [30]. [6-^{3}H]Thymidine (specific radioactivity 30 Ci/mmol), L-[^{14}C(U)]leucine (specific radioactivity 330 mCi/mmol), and L-[2,5-^{3}H]histidine (specific radioactivity 51 Ci/mmol) were purchased from Amersham International Ltd (Amersham, U.K.).

Analytical Methods

The activity of adenosylmethionine decarboxylase was assayed as in [31]. Polyamines were determined from perchloric acid extracts by the method of Seiler [32] using the solvent system of Dreyfuss et al [33]. MGBG was determined enzymically from neutralized perchloric acid extracts by the method of Seppänen et al [34] using adenosylmethionine decarboxylase isolated from baker's yeast. DNA was measured by the method of Giles and Myers [35], and protein by the method of Lowry et al [36]. The incorporation of radioactive thymidine into DNA was determined by liquid scintillation counting after hydrolysis of the samples in hot 5% trichloroacetic acid. The protein precipitate obtained after trichloroacetic acid hydrolysis was washed, dissolved in 0.1 N NaOH, and counted for radioactivity.

RESULTS

UVB irradiation enhanced DNA and protein synthesis in mouse epidermis. Both synthetic activities reached their maximum at 48 h after the irradiation (Fig 1), and the epidermal protein content was nearly doubled in 4 days (results not shown). As also described earlier by Seiler and Knödgen [11], the epidermal spermidine concentration increased 3-fold by 48 h, while changes in the other polyamines were smaller. Ornithine decarboxylase activity reached a peak at 24 h and adenosylmethionine decarboxylase was stimulated 2-fold at 48 h (results not shown).

When the animals received DFMO in drinking water for 3 days before the irradiation, and in an ointment for 2 days thereafter, the increase in epidermal putrescine and spermidine concentrations was prevented, whereas spermine concentration was somewhat increased (Table I). Topical application of MGBG decreased both spermidine and spermine levels and the combination of the 2 drugs led to a further decrease of epidermal spermidine concentration (Table I). The intracellular accumulation of MGBG was reflected by the remarkable increase of the epidermal adenosylmethionine decarboxylase activity (Table I) due to the stabilization of the enzyme molecule by MGBG binding [37].

Both DFMO and MGBG alone had a moderate inhibitory effect on epidermal DNA synthesis (Table II), but pretreatment of the animals with peroral DFMO, followed by a combined topical application of DFMO and MGBG, produced an inhibitory effect not achievable with either drug alone: the incorporation of [^{3}H]thymidine decreased to 20% of the control value (Table II).

The effect of DFMO and MGBG on epidermal protein synthesis was comparable to that on DNA synthesis, yet the extent of the inhibition produced by the drugs was smaller: the combination of DFMO and MGBG decreased the incorporation of radioactive amino acids to about half of the control value (Table III). The inhibition of protein synthesis was also reflected by a

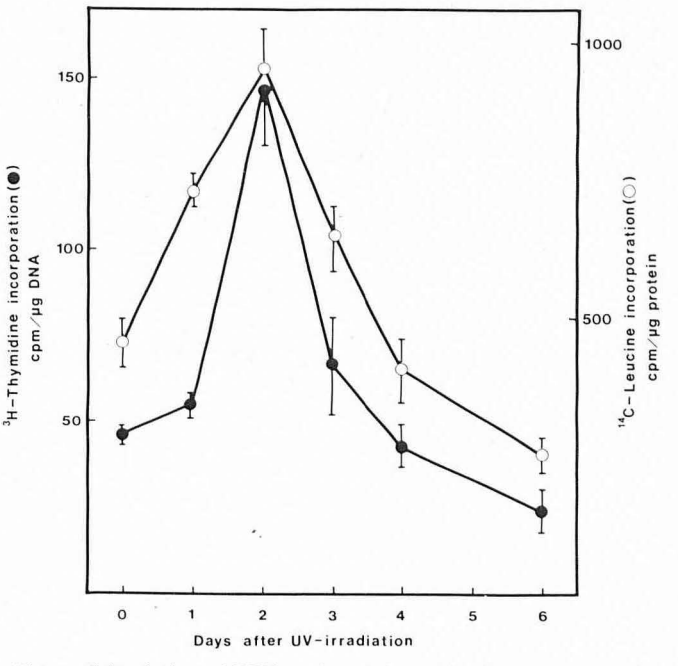

FIG 1. Stimulation of DNA and protein synthesis in mouse epidermis after UV irradiation. Each point represents the mean ± SEM of 5 animals.

TABLE I. *Effect of 2-difluoromethylornithine (DFMO), methylglyoxal bis(guanylhydrazone) (MGBG), and spermidine treatments on adenosylmethionine decarboxylase (AMDC) activity and polyamine levels in mouse epidermis*

Treatment[a]	AMDC activity[b] (nmol/mg protein/h)	Putrescine	Spermidine (nmol/mg DNA)	Spermine
None	0.7 ± 0.1[c]	77 ± 15	601 ± 63	168 ± 18
DFMO	2.4 ± 0.2[d]	10 ± 0[e]	308 ± 24[e]	193 ± 13
MGBG	32.1 ± 1.7[d]	33 ± 4[f]	326 ± 27[e]	74 ± 10[e]
DFMO + MGBG	34.1 ± 2.6[d]	87 ± 34	281 ± 34[e]	96 ± 6[e]
DFMO + MGBG + spermidine	24.0 ± 1.8[d]	112 ± 141	1097 ± 147[f]	70 ± 14[e]
Spermidine	1.6 ± 0.2[e]	88 ± 1	1239 ± 275	127 ± 18

[a] The schedule of the treatments is described in *Materials and Methods.*
[b] AMDC activity was assayed after dialysis of the samples to remove all MGBG.
[c] Mean ± SEM.
[d] $p < 0.001$.
[e] $p < 0.01$.
[f] $p < 0.05$.

TABLE II. *DNA synthesis in mouse epidermis following 2-difluoromethylornithine (DFMO), methylglyoxal bis(guanylhydrazone) (MGBG), and spermidine treatments*

Treatment[a]	[^{3}H]Thymidine incorporation (cpm/μg DNA)	DNA content (μg/mg tissue)
None	120 ± 21[b]	2.37 ± 0.10
DFMO	71 ± 8	2.44 ± 0.11
MGBG	50 ± 8[c]	2.51 ± 0.18
DFMO + MGBG	23 ± 6[c]	2.18 ± 0.07
DFMO + MGBG + spermidine	75 ± 12	2.27 ± 0.04
Spermidine	194 ± 91	2.17 ± 0.06

[a] The schedule of the treatments is described in *Materials and Methods.*
[b] Mean ± SEM.
[c] $p < 0.01$.

decrease in total protein content of the epidermal tissue (Table III).

Earlier studies by Fukuyama and Epstein [38,39] have shown that the incorporation of radioactive amino acids into different epidermal layers is not uniform, but some amino acids, e.g., leucine, preferably incorporate into basal cells, while others, such as histidine, are found in the granular layer. We labeled the epidermal proteins with [^{14}C]leucine and [^{3}H]histidine, and calculated the ratio of histidine/leucine incorporation after various treatments (Table III). Both DFMO and MGBG decreased the ratio slightly, and the decline was more pronounced after the combined treatment (Table III).

Topical spermidine treatment resulted in a 2-fold increase in the epidermal spermidine content (Table I). When given alone, spermidine appeared to have a marginal stimulatory effect on epidermal DNA synthesis (Table II), while protein synthesis remained unchanged (Table III). We have earlier shown that spermidine treatment prevents the uptake of MGBG into mouse epidermis [29], and the decrease of cellular MGBG is reflected by the cellular adenosylmethionine decarboxylase activity, as also shown in Table I. The inhibition of DNA and protein synthesis caused by combined DFMO–MGBG treatment could be partially reversed by concomitant application of spermidine: the inhibition of DNA and protein synthesis was corrected by about 55% (Tables II, III). Similarly, the histidine/leucine ratio was partially "normalized" (Table III).

DISCUSSION

Cell division seems to be strictly dependent on normal polyamine levels, and inhibitors of polyamine synthesis generally exert a distinct antiproliferative action on cultured cells and on animal tumors [14]. These observations have provoked an interest concerning the possible usefulness of polyamine antimetabolites in the treatment of neoplastic and hyperproliferative diseases. Some clinical trials have already been carried out using DFMO in the treatment of leukemia [26] and psoriasis [40]. MGBG, on the other hand, was used in the treatment of human malignancies long before its connection with polyamine metabolism was revealed [41]. Because of its narrow therapeutic index the drug was gradually withdrawn from clinical use. However, promising results recently were obtained in leukemia when the dosing of the drug was guided by carefully monitoring the MGBG levels in blood and in leukemia cells [26,42].

The chances of avoiding the side effects of MGBG were greatly increased when the concept of DFMO "priming" was discovered [27]. Pretreatment with DFMO was found to greatly increase the uptake of MGBG into cells. Moreover, since both the decrease of polyamine levels by DFMO and the uptake of MGBG is most efficient in actively dividing cells [21], the inhibitors obviously act rather selectively on neoplastic cells and other hyperplastic tissues.

Our previous studies have shown that the DFMO "priming" concept is potentially useful also in the treatment of epidermal tissue [23,29]. The present results indicate that a combined use of DFMO and MGBG elicits a profound inhibition of epidermal DNA synthesis, not achieved with either of the drugs alone (Table II).

Both DNA synthesis and cell division occur only in the basal layer of epidermis. Since DFMO and MGBG act most effectively on proliferating cells, specific accumulation of MGBG into basal cells could be expected. However, while in cell culture the drugs are directly available in the medium, in skin they must first penetrate through the upper epidermal layers, which form an effective barrier especially for polar ionizable substances such as DFMO and MGBG. The high drug concentrations used to circumvent this difficulty are likely to affect also the cells that form the differentiating surface layers of epidermis. The results in Table III suggest that the effect of MGBG on epidermal protein synthesis is in no way restricted to basal cells. The changes in the ratio of histidine/leucine incorporation indicate that the synthesis of histidine-rich keratin proteins in the granular layer is also inhibited by both DFMO and MGBG (Table III). Although the changes in the labeling may be partly due to differences in the size of the cellular amino acid pools or in the uptake of the radioactive precursors (small differences could, in fact, be observed in the acid-soluble radioactive fraction among the groups), the results clearly indicate that the topical treatment, probably owing to a rather massive accumulation of the drugs near the surface of the skin, affects the function of all epidermal cell layers.

The present study shows that DNA synthesis and, consequently, skin proliferation can be locally inhibited by a combined use of DFMO and MGBG. The combination should be potentially useful also in the treatment of human hyperproliferative skin diseases such as psoriasis, inasmuch as the systemic side effects of MGBG can be avoided by the local appli-

TABLE III. *Effect of 2-difluoromethylornithine (DFMO), methylglyoxal bis(guanylhydrazone) (MGBG), and spermidine on epidermal protein synthesis as measured by [^{14}C]leucine and [^{3}H]histidine incorporation*

Treatment[a]	[^{14}C]Leucine incorporation (cpm/mg protein)	[^{3}H]Histidine incorporation (cpm/mg protein)	Histidine/leucine ratio	Protein content (μg/mg tissue)
None	600 ± 38[b]	702 ± 111	1.17 ± 0.03	147 ± 4
DFMO	502 ± 17[c]	541 ± 63[c]	1.08 ± 0.07[c]	130 ± 4[c]
MGBG	425 ± 51[c]	437 ± 123[d]	1.03 ± 0.09[c]	141 ± 7
DFMO + MGBG	335 ± 64[e]	304 ± 133[d]	0.91 ± 0.07[d]	118 ± 2[d]
DFMO + MGBG + spermidine	492 ± 28	516 ± 71[c]	1.05 ± 0.11[c]	137 ± 8
Spermidine	553 ± 25	665 ± 39	1.21 ± 0.06	146 ± 8

[a] The schedule of the treatments is described in *Materials and Methods.*
[b] Mean ± SEM.
[c] $p < 0.05$.
[d] $p < 0.001$.
[e] $p < 0.01$.

cation technique. Furthermore, the use of polyamine antimetabolites together with other drugs for psoriasis, e.g., retinoids, could lead to a synergistic effect of both drugs. Preliminary clinical studies for the development of a practical and effective treatment schedule for polyamine antimetabolites in psoriasis are presently in progress.

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Selective Aberration and Pigment Loss in Melanosomes of Malignant Melanoma Cells in Vitro by Glycosylation Inhibitors: Premelanosomes as Glycoprotein*

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We have found that glucosamine (1 mg/ml) or tunicamycin (0.2–0.4 μg/ml), specific inhibitors of lipid carrier-dependent glycosylation of protein, when added to cultured B-16 melanoma cells produce a marked loss of pigmentation, accompanied by distinctive biochemical as well as ultrastructural aberrations in their melanogenic compartments. Electron microscopic analysis shows that these newly induced unpigmented cells form uniquely altered melanosomes containing little or no melanin, although their population is not substantially reduced. Within the melanogenic compartments, selective aberration of melanosomes is seen, that is, deformity, bulging, and segregation of their interior membrane, as well as the intramelanosomal formation of irregularly concentric lamellar structure. No apparent structural deformity of Golgi apparatus, Golgi-associated endoplasmic reticulum of lysosome (GERL), and coated vesicles has been observed. Further, no substantial alteration is seen in mitochondria or other cellular structures. Quantitative analysis of altered and nonaltered melanosomes has revealed that the ratio of altered premelanosomes to the total number increases to 44% in glucosamine-treated cells and to 99.5% in tunicamycin-treated cells, compared to 13% in the control.

Electron microscopic dopa reaction has also revealed that these altered melanosomes seem to exhibit a weakly positive or a negative dopa reaction in both glucosamine- and tunicamycin-treated melanoma cells although a number of dopa-positive altered melanosomes are still seen. However, GERL and coated vesicles show no apparent decrease in dopa reaction. It may be concluded that glycoprotein synthesis is integral to the formation of normal melanosomes and to their specific melanizing function, which could be impaired by inhibition of the synthesis of asparagin-linked mannose-containing sugar chains. This results in retrogressive changes in the premelanosomal tyrosinase during its maturation process.

In 1980, we reported much higher tyrosinase activity in the Golgi-associated endoplasmic reticulum of lysosome (GERL) and coated vesicle fraction than that in the premelanosome fraction of the pigment cells [1]. It has been found that tyrosinase synthesized by ribosomes is condensed and activated in the GERL-coated vesicle system, acquiring the capacity to form dopa-melanin in vitro. Thus, the transfer system of the activated tyrosinases into premelanosomes by the possible function of coated vesicles is presumed to be necessary for the initiation of melanogenesis in vivo.

Tyrosinase has been shown to exist in 3 forms, T_1, T_2, and T_3 in the intracellular melanogenic compartments [2–5], depending on its stage of maturation, which primarily involves its glycosylation. Further we have recently found the carbohydrate moiety of T_3 possesses melanogenically essential properties different from those of T_1, the synthesis of which is not interrupted by current glycosylation inhibitors [6]. The core sequence of N-glycosidically linked oligosaccharides is a key structure of T_3 tyrosinase for its melanization-inducing function in premelanosomes [6].

It has been shown that the carbohydrate moieties of glycoproteins in various cellular membranes have a role in the

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Abbreviations:
dopa: L-3,4-dihydroxy-phenylalanine
GERL: Golgi-associated endoplasmic reticulum of lysosome
GTA: glutaraldehyde
Glc: glucosamine
TM: tunicamycin