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Down-regulation of Epidermal Growth Factor Receptors by Dithranol

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Dithranol is highly effective in the treatment of psoriasis, but the exact mechanism of action is not known. Since persistent expression of epidermal growth factor (EGF) receptors in psoriatic epidermis is assumed to have pathogenetic significance, we have studied the effects of dithranol on EGF binding to the human epidermal cell line SCL-II. After treatment of cells with dithranol or its therapeutically inactive oxidation product, danthrone, radioligand binding assays were performed with ^{125}I-EGF. In therapeutically active concentrations (0.25–1 μg/ml) dithranol induced a decrease in EGF binding in a dosedependent manner. Danthrone was inactive. The inhibition occurred after a latency period of 6 h and reached its maximum at 24 h. At the concentration of 1 μg/ml, the drug led to approximately a 70% decrease in the number of specific high-affinity EGF receptors (Bmax), whereas receptor affinity (Kd) showed no change. The down-regulation of EGF receptors on epidermal cells by dithranol may contribute to its antipsoriatic action. *Key word: Psoriasis.*

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Dithranol (1,8-dihydroxy-9-anthrone) is a highly effective compound in the treatment of psoriasis, but despite numerous clinical and experimental investigations in recent years, its mode of activity is still not fully understood (1). Although dithranol inhibits polymorphonuclear leukocyte (PMN) function (2) and modulates the arachidonic acid metabolism (3, 4), a direct inhibition of keratinocyte proliferation has also been suggested (5). The epidermal hyperproliferation in psoriasis may be due – at least partly – to the abnormal regulation of epidermal growth factor/transforming growth factor-alpha (EGF/TGF-alpha) receptor expression (6). The EGF receptors are increased two- to fourfold in involved psoriatic epidermis and this may have pathogenetic significance (7). Recently, the antiproliferative effect of cyclosporin, TGF-beta, interferon-gamma (8, 9), and ultraviolet B (10) has been reported to down-regulate EGF receptors on keratinocytes. Since dithranol associates rapidly with the cell membrane (5) and is capable of modulating cell surface receptors of epidermal cells (11), we were interested in seeing if dithranol influences epidermal EGF receptors. Therefore, we studied the effects of dithranol and its therapeutically inactive oxidation product, danthrone, on EGF receptors in the human epidermal cell line SCL-II. Here we show that in contrast to danthrone, dithranol causes a pronounced down-regulation of EGF receptors on epidermal cells. This may contribute to its antipsoriatic action.

MATERIALS AND METHODS

Chemicals

Dithranol (1,8-dihydroxy-9-anthrone) and danthrone (1,8-dihydroxyanthraquinone) were kindly provided by Dr. Schmersahl, Hermal Chemie (Reinbek, Germany). The compounds were always freshly dissolved in acetone (10 mg/ml), then diluted in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% fetal calf serum (FCS), kept protected from light, and used immediately. DMEM and FCS were obtained from Flow Laboratories (Meckenheim, Germany). ^{125}I-EGF, specific activity 2200 Ci/mmol, was purchased from Amersham (Braunschweig, Germany), unlabelled EGF from IC Chemicalien (München, Germany).

Cell culture

SCL-II cells were derived from an explant culture of a human squamous cell carcinoma (12) and kindly provided by Prof. N.E. Fusenig (German Cancer Research Center, Heidelberg, Germany). Cells were cultured at 37°C in a humid 5% CO_2 atmosphere in DMEM containing 2 mM glutamine (Flow Laboratories, Meckenheim, Germany), 100 U/ml penicillin (Gibco, Eggenstein, Germany), 25 μg/ml streptomycin (Gibco, Eggenstein, Germany), and 10% heat-inactivated FCS. After reaching confluency, cultures were routinely passaged with 0.1% trypsin/0.02% EDTA in phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++}. The radioligand binding assays were performed on subconfluent monolayer cells that were transferred to 24-multiwell-tissue culture plates (2×10^5 cells/well) and studied 24 h later.

Ligand binding assays

The subconfluent monolayer SCL-II cells were washed twice with PBS under sterile conditions and incubated for 1 h at 37°C with increasing concentrations of dithranol or danthrone (0.1–1.0 μg/ml) in DMEM containing 0.1% FCS. Higher concentrations of dithranol than 1 μg/ml caused a decrease (30%) in the number of cells per well as compared with solvent-treated controls. Therefore, 1 μg/ml was the highest dithranol concentration used in the present experiments, which is also relevant to the concentrations present in the epidermis during treatment with the drug (13). After treatment cells were washed 4 times with PBS and cultured for 24 h at 37°C in culture medium with 0.5% FCS. In a standard binding assay SCL-II cultures were washed twice with PBS, cooled on ice and then incubated with 0.2 nM ^{125}I-EGF in DMEM with 0.2% bovine serum albumin (Sigma Chemical Co, Germany) and buffered with 10 mM HEPES (400 μl/well) at 4°C. After 4 h, the assay was terminated by washing the monolayers 3 times with icecold PBS. Cells were then solubilized in 300 μl 0.1 N NaOH, and cell-bound radioactivity was counted in a gamma counter. Nonspecific binding was determined by adding a 100-fold excess of unlabelyed EGF to parallel sample wells. It was not higher than 5% of the total bound radioactivity under any treatment conditions. The specific binding was obtained by subtracting nonspecific from total ligand binding. The cell number was counted for every well, and the data were expressed as specifically bound ^{125}I-EGF in fmol/10^5 cells. Each assay was carried out in duplicate. Cell viability was determined by the trypan blue exclusion test and was greater than 90% for all dithranol concentrations.

The time kinetics of dithranol-induced inhibition of EGF binding was studied by incubating cells for 1 h with or without dithranol (1

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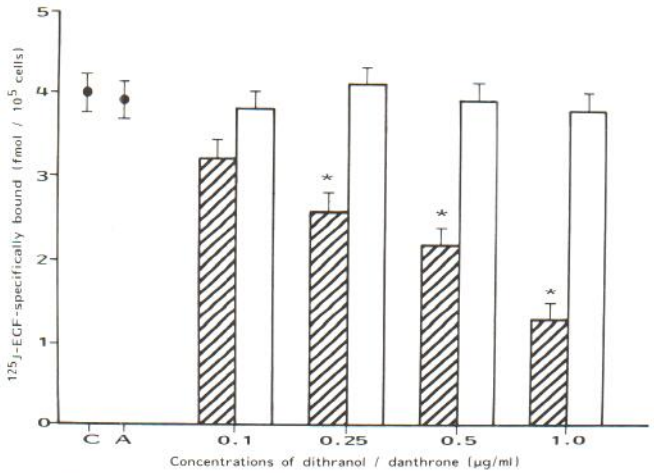

Fig. 1. Dose-dependence of dithranol-induced inhibition of EGF binding in SCL-II cells. Radioligand binding assays were performed 24 h after the treatment as described in Materials and Methods. *C*: control data for DMEM, *A*: for the solvent acetone 0.1% (v/v). ▨: dithranol, □: danthrone: Values represent means ± SD for three experiments performed in duplicate. *: $p < 0.05$ as compared to the control.

µg/ml) in DMEM with 0.1% FCS. Cells were then washed with PBS, the culture medium containing 0.5% FCS was replaced, and the radioligand binding assays were performed 1, 6, 12, 24 and 48 h later as described above.

The cells were incubated for 1 h with 1 µg/ml dithranol or 1 µg/ml danthrone. Control cells were treated with the solvent alone. After the cells had beem washed. the culture medium containing 0.5% FCS was replaced, and 24 h later binding assays were performed by incubating cells with increasing concentrations of ^{125}J-EGF in the range of 0.05–0.8 nM in a final volume of 400 µl/well. Nonspecific binding was determined in the presence of a 100-fold excess of unlabelled EGF. Saturation curves were analyzed for EGF receptor number (Bmax) and affinity (Kd) using a computerized nonlinear curve fitting program (MxN-FIT) (14).

The statistical significance of the data was calculated by using Student's *t*-test and analysis of variance.

RESULTS

Dose – response studies of the effects of dithranol and danthrone on specific EGF binding on epidermal cells

Fig. 1 shows the dose-dependence of dithranol-induced inhibition of ^{125}I-EGF binding, when the binding assay was performed 24 h after the treatment. Data are expressed for each dose of dithranol as specifically bound ^{125}I-EGF per cell number. Dithranol induced a dose-dependent decrease in specific ^{125}I-EGF binding to epidermal cells ($p<0.05$, analysis of variance). Significant inhibition of EGF binding was observed even at 0.25 µg/ml concentration of dithranol and reached a maximal level of approximately 70% compared to solvent-treated controls at 1 µg/ml of dithranol. Danthrone had no effect on EGF binding at the same concentrations. Nonspecific binding remained unaffected after treatment of cells with dithranol or danthrone (data not shown). Furthermore, the dithranol concentrations used had no effect on cell viability as compared to the controls.

Time – course studies of the effects of dithranol on EGF binding

To study the time kinetics of dithranol-induced decrease of EGF binding, cells were treated with 1 µg/ml of dithranol for 1 h, and radioligand binding assays were then performed at varying time intervals, i.e. 1, 6, 12, 24 or 48 h. In Fig. 2 data are expressed as a percentage of specific EGF binding in comparison to the solvent-treated controls. The results show that the dithranol-induced inhibition of specific EGF binding occurred after an initial latency period of 6 h, with maximal effect at 24 h, slowly reversing after that (Fig. 2).

Analysis of the effects of dithranol on EGF receptor number and affinity

In order to further analyse the kinetics of dithranol-induced inhibition of EGF binding, radioligand binding assays were performed with increasing concentrations of ^{125}I-EGF 24 h after treatment of cells with 1 µg/ml dithranol, 1 µg/ml danthrone, or with the solvent alone. The Kd and Bmax values were obtained by batch analysis of the saturation isotherms of three independent experiments with a nonlinear curve-fitting program (MxN-FIT) (14). Control SCL-II cells showed the presence of a single class of high affinity binding sites for EGF with a Kd of 0.21 nM and a Bmax of 48000 receptors per cells. Treatment of cells with 1 µg/ml dithranol resulted in a decrease in receptor number (14200 receptors/cells), while the Kd value remained unchanged (0.19 nM). Danthrone had no effect on Bmax and Kd values (Fig. 3).

DISCUSSION

In the present work we have studied the effects of dithranol and its therapeutically inactive oxidation product, danthrone, on the EGF receptors in a human keratinocyte cell line, SCL-II. We have found that treatment of the cells for 1 h (a contact time sufficient to exert an antiproliferative effect in vitro) (5) with dithranol induces a marked decrease in EGF binding in a dose-dependent manner. The therapeutically in-

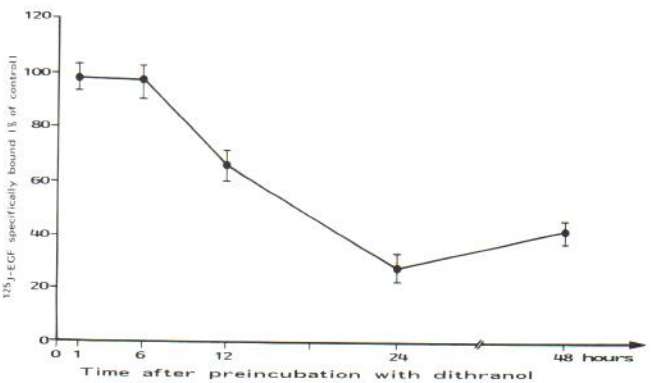

Fig. 2. Time course of dithranol-induced inhibition of EGF binding in SCL-II cells. Cells were treated with 1 µg/ml of dithranol and binding assays were then performed at varying time points as described in Materials and Methods. Data are expressed as percent of control specific binding. Points represent means ± SD for three experiments performed in duplicate.

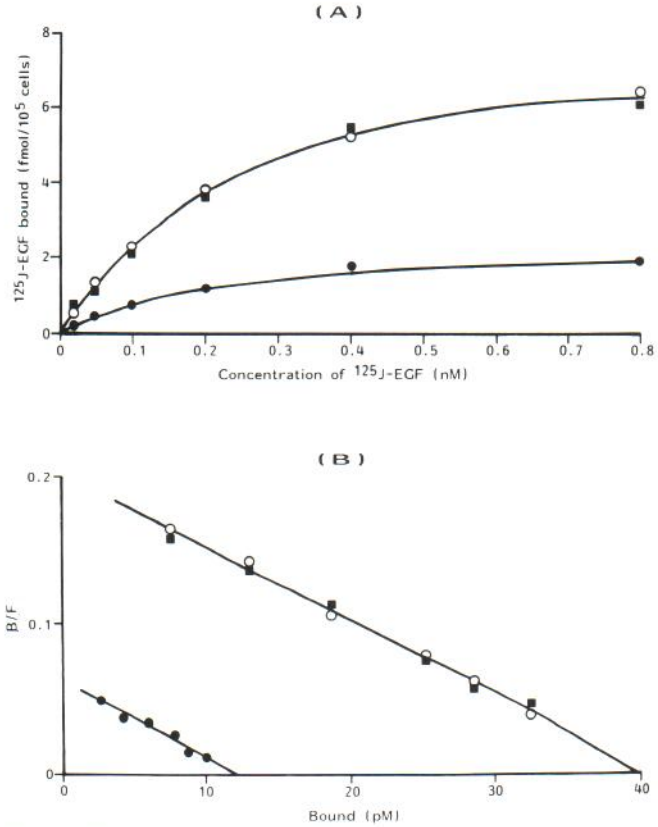

Fig. 3. Effect of dithranol on EGF receptor number (Bmax) and dissociation constant (Kd) in SCL-II cells. 24 h after treatment with 1 μg/ml dithranol (●), 1 μg/ml danthrone (■) or with the solvent (○); binding assays were performed as described in Materials and Methods (A). Scatchard plots of the data (B). Each point indicates the mean of duplicate samples and is representative of three independent experiments.

active metabolite danthrone had no effect on the binding. The inhibition of EGF binding in SCL-II cells by dithranol occurred after a latency period of 6 h and reached its maximum at 24 h, after which it declined. Analysis of saturation curves revealed that the dithranol-induced inhibition of EGF binding was due to an approximately 70% decrease in the number of EGF binding sites on SCL-II cells, whereas receptor affinity remained unchanged.

Dithranol has been reported to cause a variety of changes in cells, such as peroxidation of cell membrane through free radical formation (16). However, the observed inhibitory effect of dithranol on EGF binding to epidermal cells could not be explained by a toxic effect on cells, since cell viability was virtually unaffected by the drug at the concentrations used. Furthermore, receptor affinity remained unchanged, suggesting that an oxidant effect of dithranol on receptor protein is unlikely. The lag period before inhibition of EGF binding became evident also excludes a direct interaction between dithranol and EGF receptors. Since dithranol inhibits protein synthesis in keratinocytes in vitro through affecting mitochondrial respiration (5), the most likely explanation for the decrease in EGF binding sites is an inhibition of receptor protein synthesis. The marked decrease of Bmax of 70% and the time kinetics of the inhibition strengthen this assumption. Another potential mechanism of the observed decrease in EGF binding may be due to an altered receptor regulation. This scenario is based on the finding that dithranol activates protein kinase C (PKC) (17), and PKC activation leads to direct down-regulation and internalization/degradation of the EGF receptor through phosphorylation of key amino acid residues (18, 19).

EGF is a potent mitogen for cultured human keratinocytes. All known effects of EGF are mediated through binding to its specific receptor. In the psoriatic hyperproliferative epidermis, a two- to fourfold increase in EGF receptor number is supposed to play a role in the pathomechanism of the disease (7). Recently, various antiproliferative molecules, such as cyclosporin, TGF-β, interferon-gamma or UV-B have been reported to decrease the number of EGF receptors (8–10). Our finding that dithranol also induces a decrease in EGF receptor number in epidermal cells supports the concept that the down-modulation of EGF receptors may be a general mechanism of action of antiproliferative molecules.

After submission of this work, the dithranol-induced inhibition of EGF binding to cultured normal human keratinocytes was reported in another paper (20), which reached conclusions concerning the inhibitory effect of dithranol very similar to these found here.

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