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The antipsoriatic agent dimethylfumarate immunomodulates T-cell cytokine secretion and inhibits cytokines of the psoriatic cytokine network

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Summary Interactions between infiltrating T cells and keratinocytes via the secretion of the TH1 cytokines interleukin (IL) 2 and interferon γ (INF-γ), the keratinocyte growth factor transforming growth factor α (TGF-α) and the cytokines IL-6 and IL-8 are thought to be the predominant mechanisms inducing skin lesions in psoriatic patients. Systemic treatment of psoriasis with fumaric acid derivatives (FAEs) has been reported to be effective in the treatment of psoriasis, but the mode of action is still unknown. To clarify this phenomenon, keratinocytes from psoriatic patients as well as from healthy volunteers were mono- and cocultured with HUT 78 T cells with/without the addition of FAEs; the cytokine concentrations were then measured in the culture supernatants. Furthermore, mRNA expression was determined in epidermal growth factor (EGF) -activated keratinocytes as well as in phytohaemagglutinin (PHA)-activated HUT 78 T cells. Only dimethylfumarate (DMF) diminished IL-6 and TGF-α secretion in the psoriatic cocultures. However, it did not have this effect on cocultures from control subjects or on monocultures. DMF suppresses EGF-induced TGF-α mRNA induction in psoriatic keratinocytes. DMF inhibited INF-γ secretion in all cultures but stimulated the IL-10 secretion. This immunomodulation away from the TH1 cytokine IFN-γ to the TH2 cytokine IL-10 was confirmed in HUT 78 T cells by Northern blot analysis. An increased number of eosinophils is a known side-effect in patients treated with this drug, suggesting a clinical relevance of this immunomodulation *in vivo*. This immunomodulation and the suppression of cytokines from the psoriatic cytokine network could be responsible for the beneficial effect of DMF in the treatment of a hyperproliferative and TH1 cytokine-mediated skin disease.

Psoriasis is a common inflammatory cutaneous disorder with a prevalence of 2–3% in the general population. Recent findings support a central role for immunological mechanisms in the aetiology of psoriasis. Early psoriatic lesions are predominantly infiltrated by CD4 + T cells. These T cells produce high amounts of the so-called T-helper 1 (TH1) cytokines interferon γ (IFN-γ) and interleukin (IL) 2 and of other inflammatory cytokines (e.g. IL-6). The effectiveness of the immunosuppressive drug cyclosporin A or of anti-CD4 monoclonal antibodies in the treatment of psoriasis underlines the central role of immunocompetent T cells in the pathogenesis of this disease.[1–3]

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The paper contains data from the doctoral thesis of Gabriele Ockenfels. Some of this work was presented in a lecture given at the congress 'Psoriasis: from gene to clinic', London, 5–7 December 1996.

In addition to these TH1 cytokines, the proinflammatory cytokines IL-6 and IL-8, as well as the keratinocyte growth factor (transforming growth factor alpha; TGF-α), are found to be highly elevated in the psoriatic skin lesion. Consequently, an idea prevalent among the hypotheses proposed to explain the inflammatory and hyperproliferative psoriatic skin lesions is that keratinocytes and skin-infiltrating T cells elaborate a specific cytokine network including these five mentioned cytokines.[2,4] In some western European countries, a new (old) systemic therapy of psoriasis with fumaric acid esters (FAEs) is increasingly playing a major part in the treatment of psoriasis. More than 30 years have passed since the biochemist Schweckendick[5] reported the successful treatment of his own psoriatic lesions with fumaric acid.

Although fumaric acid was widely used outside the field of dermatology for more than 20 years, FAEs have now been reintroduced in the dermatological clinics,

and many double-blind, placebo-controlled studies have proved the efficacy of these.[6] The current fumaric acid therapy for psoriasis is the oral administration of dimethylfumarate (DMF) combined with salts (magnesium, calcium and zinc) of monoethyl fumarate (MEF). Because almost nothing was known about the antipsoriatic mechanisms of FAEs at the cellular level, we investigated the effect of FAEs on the psoriatic cytokine network. Therefore, keratinocytes were mono- or cocultured in a recently described coculture model[7,8] with/without FAEs and cytokine mRNA induction in the cells; cytokine levels in the culture supernatants were also determined.

Materials and methods

Patients

Skin biopsies were taken from the lesional plaques of nine patients with psoriasis (six men and three women; mean age 45 years, range 23–65 years) and from 14 healthy volunteers (nine men and five women; mean age 53 years, range 25–75 years), who served as the control group. All the patients had severe psoriasis with more than 20% of the skin surface affected. None of these patients had received any topical (e.g. calcipotriol or dithranol) therapy 6 weeks before or systemic immunosuppressive therapy 6 months before the study.

Cocultures of keratinocytes with HUT 78 T cells

Keratinocyte cultures were performed with standard methods in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) in view of the coculture conditions. In passages 4–5, keratinocytes were cocultivated with HUT 78 T cells as described previously.[7,8] HUT 78 T cells (ATCC, Rockville, MD, U.S.A.) are a continuously activated T-cell lymphoma cell line growing in RPMI-1640 supplemented with 10% FCS.[9] HUT 78 T cells are known to be capable of binding to non-cytokine-pretreated keratinocytes and were first described in coculture with keratinocytes by Stoof *et al.*[9] in 1992. These cells are known to produce cytokines of the TH1 group (IL-2 and IFN-γ) as well as cytokines of the TH2 group (IL-4, IL-5 and IL-10).[8,10,11]

Briefly, keratinocytes (10^5/mL) were seeded in 24-well flat-bottomed microtitre plates. After 2 days, the medium was removed, and HUT 78 T cells (10^5/mL) were placed in an insert well (Millipore, 0·45 μm pore size) 2 mm above the top of the keratinocytes with a final volume of 2 mL of RPMI + 10% FCS. After 24 h, half the medium was removed. FAEs (DMF, Ca-MEF, Mg-MEF or Zn-MEF) were added to the cultures at final concentrations of 5 and 30 μmol/L (Fumapharm, Switzerland) over 24 h, and the culture supernatants were collected. HUT 78 T cells were washed out from the insert well with phosphate-buffered saline (PBS), and keratinocytes were trypsinized with 0·5% trypsin and 0·05% EDTA. The cell numbers were determined in a Neugebauer counting chamber, and the viability of the cells was analysed by Trypan blue exclusion.

Monocultures

Corresponding to these cocultures, monocultures and experiments with FAEs were performed over the same time period, and the cytokine contents were measured in culture supernatants. Additionally, HUT 78 T cells (10^5/mL; $n = 5$) were monocultivated with another stimulus, in this case with the mitogen phytohaemagglutinin (PHA; 5 μg/mL), and IL-10 and IFN-γ concentrations were determined after 2, 4, 8 and 12 h in the culture supernatants. At this time point, total RNA was isolated from the HUT 78 T cells ($n = 4$), and Northern blot analysis for IL-10 and IFN-γ was done as follows. Similarly, to confirm the inhibition of TGF-α secretion in psoriatic cocultures by DMF, keratinocytes from psoriatic patients and from control subjects ($n = 5$) were activated with epidermal growth factor (EGF; 5 ng/mL), and the effect of DMF on TGF-α mRNA expression was analysed by Northern blotting.

Cytokine detection in culture supernatants

The culture supernatants were collected, and the cytokine concentrations of IL-6, IL-8, IL-10, IFN-γ and TGF-α were measured using standard enzyme-linked immunosorbent assay (ELISA) techniques. ELISA kits for IL-8 and IL-10 were obtained from Medgenix (Ratingen, Germany), for IL-6 and TGF-α from Immunotech (Staufenberg, Germany) and for IFN-γ from Laboserv (Hamburg, Germany). The cytokine concentration was standardized to 2×10^5 lymphocytes or keratinocytes in the monoculture and to 4×10^5 cells in the coculture system after the density and viability of keratinocytes and HUT 78 T cells had been analysed. The sensitivity of the ELISAs as well as the necessary cell numbers for the detection of cytokines in these systems were tested during the establishment of the mono- and coculture system. Statistical significance was determined using the Wilcoxon–Mann–Witney test.

Table 1. Cytokine concentrations in mono- and cocultures of keratinocytes from psoriatic patients and control subjects and HUT 78 T cells with/without the addition of fumarates over 24 h. Psoriatic (KC Pso) and control (KC Con) keratinocytes and HUT 78 T cells (HUT 78) were monocultured. Psoriatic (KC Pso + T) or control (KC Con + T) keratinocytes were cocultivated with HUT 78 T cells. DMF or Mg-MEF were added at 5 μmol/L. Mean ± SEM.

	IL-6			TGF-α			IFN-γ			IL-10		
	Without	+ DMF	+ Mg-MEF	Without	+ DMF	+ Mg-MEF	Without	+ DMF	+ Mg-MEF	Without	+ DMF	+ Mg-MEF
Kc Pso	241 ± 65*	202 ± 49	195 ± 41·5	208 ± 59·7*	174 ± 35·4	197 ± 42·3	–	–	–	< 5	< 5	
KC Con	126 ± 37·9	143 ± 29·8	147 ± 31·9	97 ± 22·1	127 ± 29·8	132 ± 19	–	–	–	< 5	< 5	
HUT 78	34 ± 4·6	38 ± 5·1	32 ± 7·8	–	–	–	83 ± 9·1	42·3 ± 5**	66 ± 11·2	208 ± 19·5	272 ± 25·3**	203 ± 10·2
KC Pso + T	277 ± 52*	146 ± 24**	293 ± 24	310 ± 44·3*	164·9 ± 21·2**	341 ± 26	139 ± 11	90 ± 14·1**	137 ± 9·1	260 ± 30·8	340 ± 28·9**	273 ± 23·2
KC Con + T	125 ± 19·1	142 ± 17·8	135 ± 18·3	106 ± 23·5	133 ± 26·5	113 ± 21·3	155 ± 19·3	96·1 ± 10·2**	140 ± 10·4	276 ± 31	345 ± 28·5**	289 ± 19·9

*Cytokine levels were elevated in psoriatic cultures ($P < 0{\cdot}05$). **DMF significantly altered the cytokine secretion compared with DMF-untreated cultures ($P < 0{\cdot}05$); *P*-values based on Wilcoxon–Mann–Witney test.

Northern blot analysis

In order to correlate the results of the protein level assays, Northern blot analysis of IL-10, IFN-γ and TGF-α mRNA levels in keratinocytes and/or in HUT 78 T cells was performed. Total RNA was isolated both from monocultured keratinocytes (with/without stimulation by EGF, 5 ng/mL) and from HUT 78 T cells according to established procedures. For Northern blotting, denatured total cellular RNA was separated by gel electrophoresis using 1% agarose gels containing 6·3% formaldehyde and 1 × MOPS. Gels were run at 85 V for 2 h and subsequently transferred to a nylon filter by capillary blotting. Filters were baked at 80 °C for 2 h. After prehybridization according to standard protocols, hybridization was conducted using deoxyadenosin-5′-[^{32}P]triphosphate-labelled cDNA probes encoding specifically for TGF-α, IL-10 and IFN-γ (ATCC). cDNA probes were labelled by random priming. After two washes, hybridized membranes were exposed on Kodak X-OMAT AR films at − 80 °C. For purposes of comparison, the background was subtracted from the cytokine signals and the glyceraldehyde-3-phosphate dehydrogenase signals (serving as a control for RNA loading), and the ratio of their intensities was calculated using Scan Analysis software.

Results

As expected,[1–3,7,8] we measured higher cytokine levels of IL-6 and TGF-α in the psoriatic mono- and cocultures ($P < 0{\cdot}05$) than in the cultures with control keratinocytes (Table 1). The cocultivation of HUT 78 T cells with keratinocytes did not alter the cytokine concentrations compared with monocultures, with the exception that the IFN-γ secretion of HUT 78 T cells was raised above 50% under coculture conditions. No IFN-γ and no IL-10 could be measured by ELISAs in the monocultures of keratinocytes; moreover, no TGF-α was measured in the HUT 78 T cell monocultures.

The IL-8 concentration was not significantly altered by FAEs (data not shown). By comparison with the salts of MEF, only the addition of DMF exclusively altered the cytokine levels in the cell cultures. The addition of DMF, even at a concentration of 5 μmol/L, diminished TGF-α and IL-6 concentrations only in the psoriatic cocultures ($P < 0{\cdot}005$). We also detected a reduction in the cytokine concentration in the psoriatic monocultures but not in the monocultures of control subjects. However, this effect was not significant ($P < 0{\cdot}1$). These data are presented in Table 1. In Table 1, Mg-MEF represents the

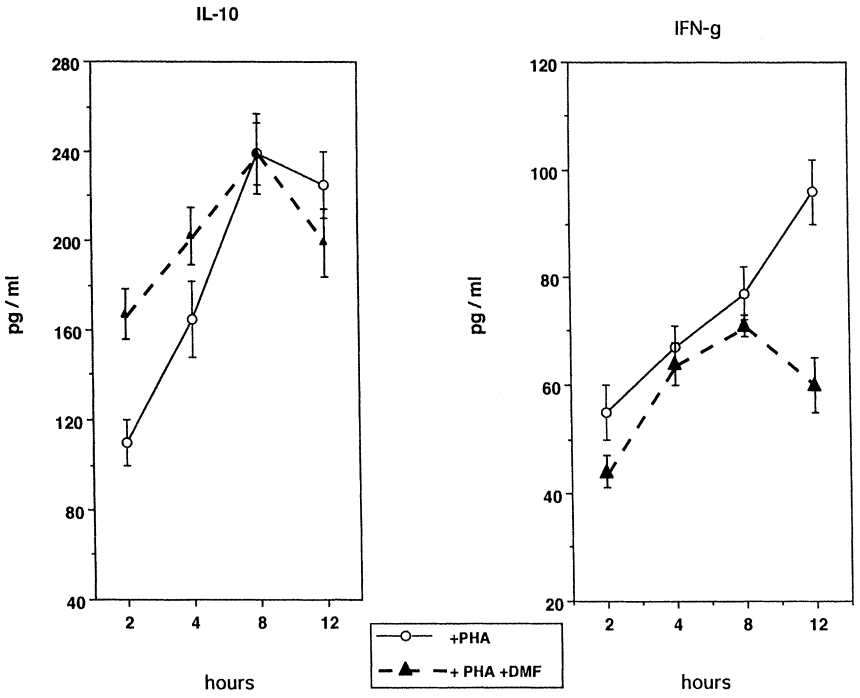

Figure 1. Effect of dimethylfumarate (DMF) on cytokine secretion of phytohaemagglutinin-stimulated HUT 78 T cells over 2, 4, 8 and 12 h. Values are given in pg/mL/10^5 cells. $n = 5$; DMF = 5 μmol/L final concentration. Differences in interleukin-10 secretion after 2 and 4 h ($P < 0{\cdot}002$; $P < 0{\cdot}05$) and interferon-γ secretion after 2 and 12 h ($P < 0{\cdot}05$; $P < 0{\cdot}002$) are significant.

data of the salts of MEF. Mg-MEF also distinctly reduced the cytokine concentration of IL-6 and TGF-α in the monocultures of psoriatic keratinocytes ($P < 0{\cdot}1$), but this effect was not observed in the psoriatic cocultures (in contrast to DMF). All other variations in the cytokine values obtained without the addition of MEFs are not significant. Using Northern blot analysis, we found that DMF, but not the salts of MEF, impressively suppresses EGF-induced TGF-α mRNA expression in psoriatic keratinocytes. This effect was not observed in control or in unstimulated keratinocytes (data not shown).

In contrast to the DMF-induced specific downregulation of IL-6 and TGF-α concentration in psoriatic cocultures, DMF influenced the INF-γ and IL-10 content in both psoriatic and control cocultures. DMF reduced the IFN-γ content in all HUT 78 T cells containing cultures, including unstimulated HUT 78 monocultures (Table 1). Additionally, the IFN-γ concentration in PHA-stimulated HUT 78 T cells was inhibited even by 5 μmol/L DMF (Fig. 1). In contrast to this inhibitory effect of DMF on the TH1 cytokine IFN-γ, the concentration of the TH2 cytokine IL-10 was increased in HUT 78 T cells containing cocultures after 24 h (Table 1). This could be based on a very early stimulatory effect of DMF on IL-10 secretion, because we found a maximum of DMF-induced IL-10 triggering in PHA-stimulated HUT 78 T cells after 2 h (Fig. 1). This immunomodulatory effect of DMF on the T-cell cytokine secretion was confirmed by Northern blot analysis, as shown in Fig. 2(a,b).

Discussion

In this study, we found that the antipsoriatic agent DMF suppresses cytokines of the psoriatic cytokine network (IL-6 and TGF-α) and immunomodulates secretion of the T-cell cytokines IFN-γ and IL-10 *in vitro*. The so-called 'fumaric acid therapy' of psoriasis is a complex dynamic treatment and consists of the oral administration of DMF starting at a dosage of 30 mg once daily and increasing first to 120 mg daily after 3 weeks and then to 240–720 mg/day in the next weeks. The treatment includes the fixed combination of DMF (120 mg tablet) with the above-mentioned salts of MEF (95 mg tablet). Our investigations confirm the conclusion reached by other investigations, namely that DMF, which is metabolized to monomethylfumarate (MMF), is apparently the most potent antipsoriatic substance in this mixture.[12,13] DMF offers antiproliferative activity on human keratinocytes even at concentrations (> 4 μmol/L) three times lower than MEF.[13]

At the concentration used, DMF and the salts of MEF did not influence the viability of keratinocytes and T cells. This is not surprising, because the toxic effects of fumarates on cell viability have been described for > 12 μmol/L DMF and for > 35 μmol/L Mg-MEF.[13–15]

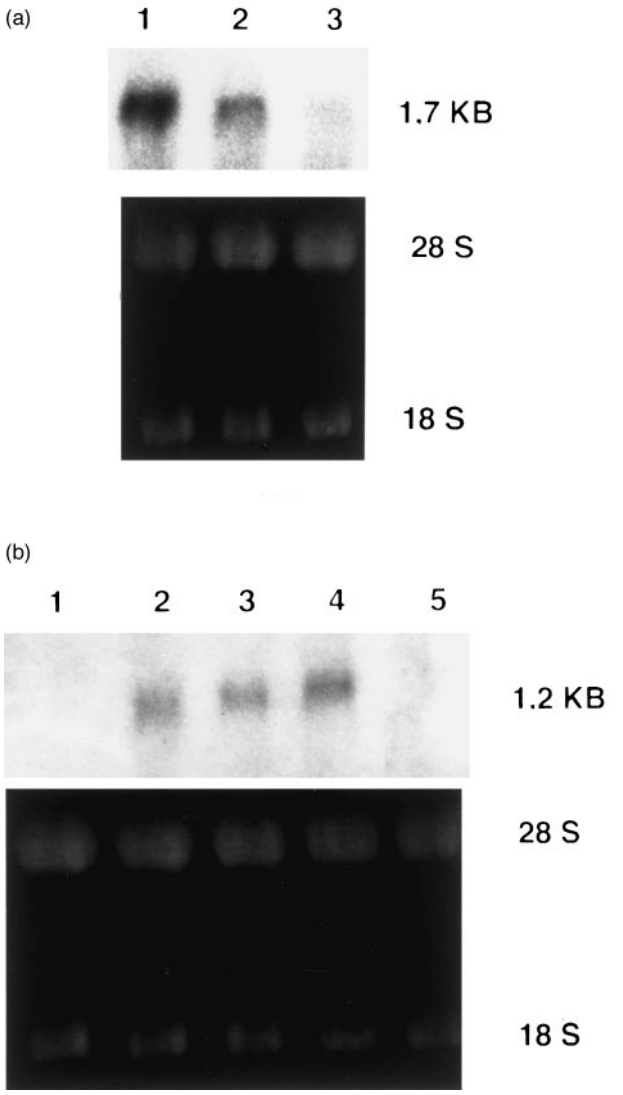

Figure 2. Cytokine mRNA expression in dimethylfumarate (DMF)-treated HUT 78 T cells. (a) Induction of interleukin (IL)-10 mRNA after 2 h in DMF-treated (5 μmol/L; lane 1), phytohaemagglutinin (PHA)-activated (lane 2) HUT 78 T cells. Lane 3 represents the IL-10 mRNA level in HUT 78 T cells without the addition of PHA and DMF. (b) Suppression of interferon (IFN)-γ mRNA by DMF (5 μmol/L; lane 1) after 4 h in PHA-activated HUT 78 T cells (lane 4). Lane 5 represents the IFN-γ mRNA level in unactivated HUT 78 T cells. No inhibitory effect of Ca-monethylfumarate (MEF) (lane 2) or of Mg-MEF (lane 3) on the IFN-γ mRNA induction even at 30 μmol/L could be detected.

For fibroblasts, IC_{50} values were characterized between 10 and 30 μmol/L DMF.[15] In contrast to these data, de Jong *et al.*[16] did not observe an inhibitory effect of the proliferative response of < 200 μmol/L MMF on T cells. Moreover, DMF monotherapy has been shown in a clinical study to be more effective than the mixtures of MEFs.[17] However, despite the *in vitro* data obtained so far and the side-effects observed in the clinical treatment of patients with FAEs (e.g. gastrointestinal complaints, nephrotoxicity, eosinophilia and lymphopenia) little was known about this drug.[6,18]

To investigate putative FAE-induced interactions between keratinocytes and T cells via their secretion of different cytokines, the *in vivo* situation was modulated by cocultivating keratinocytes with the stimulatory neighbourhood of HUT 78 T cells. Indeed, HUT 78 T cells are not representative of the TH1 T cells that are found in the psoriatic skin lesions, because they are able to produce both TH1 as well as TH2 cytokines.[8–11] If an imbalance of TH1–TH2 cytokines is created by genetic determination and trigger factors, a suppression of TH1 cytokines, as performed by drugs such as cyclosporin A, as well as an augmentation of TH2 cytokines could be desirable therapeutic mechanisms in the treatment of this disease.[2] Analysing the FAE-induced cytokine secretion in a coculture model, we chose the HUT 78 T cells with their various cytokine profiles as the favoured cell line for another time.[7,8]

In this sensitive model, we detected a reduced cytokine secretion of IL-6 and of TGF-α only in the cocultures with psoriatic keratinocytes but not with control keratinocytes or in unstimulated monocultures.

TGF-α is strongly secreted by lesional psoriatic keratinocytes and is thought to be one of the most important cytokines provoking the hyperproliferation of psoriatic keratinocytes via an autocrine stimulation.[2,4] Therefore, we focused on this cytokine and confirmed the coculture data by finding a suppressive effect of DMF on EGF-induced TGF-α mRNA induction in psoriatic keratinocytes. So, the described antiproliferative effect of DMF on highly proliferative keratinocytes[12,13] could be focused on a downregulation of TGF-α cytokine secretion in psoriatic keratinocytes to values similar to those measured in healthy control subjects (Table 1). In contrast to TGF-α, the cytokine IL-6 can be secreted by keratinocytes as well as by HUT 78 T cells.[7,8,19] Because DMF induced only a diminished IL-6 level in psoriatic cocultures, we speculated that DMF inhibits this cytokine secretion in psoriatic keratinocytes or inhibits other cytokine secretions of these interactive cells and influences IL-6 secretion via paracrine effects between psoriatic keratinocytes and HUT 78 T cells.

Furthermore, and these are the most surprising data, DMF immunomodulates cytokine secretion away from the TH1 cytokine IFN-γ to the secretion of the TH2 cytokine IL-10. First, we detected this effect in our coculture model and then demonstrated a time-dependent influence of IL-10 and IFN-γ mRNA expression in PHA-stimulated HUT 78 T cells by DMF. Recently, a

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