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Dimethylfumarate Is an Inhibitor of Cytokine-Induced E-Selectin, VCAM-1, and ICAM-1 Expression in Human Endothelial Cells

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Most studies on the antipsoriatic mode of action of dimethylfumarate focused on its antiproliferative effects in keratinocytes. Because inflammatory skin diseases are associated with an upregulation of endothelial cell adhesion molecules and because the presence of inflammatory cells in dermis and epidermis is considered an important feature in psoriasis, we tested the effect of DMF on cytokine-induced adhesion molecule expression in HUVEC, using *in situ* ELISA and Northern blotting. Dimethylfumarate inhibited ICAM-1, VCAM-1, and E-selectin expression and reduced adhesion of U937 cells to stimulated HUVEC. Monoethylfumarate and fumaric acid had no effect. Similar inhibitory effects for DMF on VCAM-1 expression were observed after stimulation of HUVEC with LPS, PMA, IL-4, and IL-1α or in combinations with TNFα. These data are in agreement with previously reported effects of DMF on intracellular thiol levels and inhibition of NF-κB activation. The inhibitory effect on cytokine-induced endothelial adhesion molecule expression may represent another target of dimethylfumarate in psoriasis.

The recruitment of leukocytes from the blood into the peripheral tissues in inflammation is mediated through the concerted action of different leukocyte-endothelium cell adhesion molecules in response to various stimulatory factors (1-4). On endothelial cells (EC) intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin are involved (1). VCAM-1 and E-selectin are absent on resting endothelium (5,6), ICAM-1 is present at low level (7). Upon release of cytokines a proinflammatory phenotype is induced in the endothelium in which a wide variety of genes, including those encoding for ICAM-1, VCAM-1 and E-selectin, are transcribed (8). Selectins are thought to be responsible for the 'rolling' of leukocytes along the blood vessel wall, caused by transient and reversible interactions. ICAM-1 and VCAM-1 are required for a more stable adhesion, which enables the leukocytes to transmigrate the endothelium. The leukocyte is then guided to the site of infection by a gradient of chemoattractants. Later on, the expression levels of the different adhesion molecules decline to their original values (1).

Inflammatory skin diseases such as psoriasis, atopic dermatitis and allergic contact dermatitis are associated with the epidermal and dermal accumulation of lymphocytes (9,10). Immunohistochemical studies reveal upregulation of various cell adhesion molecules on endothelial cells as well as on keratinocytes (11-13). Inhibition of extravasation at the site of the endothelium by blocking upregulation of cell adhesion molecules, diminishes infiltration of leukocytes in the (epi)-dermal tissues and reduces the subsequent inflammatory signals (14-16).

Dimethylfumarate (DMF) is an active component in Fumaderm, a marketed antipsoriaticum. In keratinocytes (KC), DMF has been shown to have an antiproliferative effect, which might be linked to a transient [Ca^{2+}] elevation (17). Keratinocyte differentiation seemed to be affected as well (18). Besides, an inhibitory effect of DMF on ICAM-1 expression on a keratinocyte cell line, HaCat cells, has been reported (19).

The present study deals with the observation that

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Abbreviations used: DMF, dimethylfumarate; FAcid, fumaric acid; hARP, human acidic ribosomal protein; hDMVEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL-1α, interleukin-1 α; IL-4, interleukin-4; LPS, lipopolysaccharide; MEF, monoethylfumarate; NF-κB, nuclear factor-kappaB; PMA, phorbol methyl ester; TNFα, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

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DMF diminishes the expression of ICAM-1, VCAM-1 and E-selectin on macro- and microvascular endothelial cells.

MATERIAL AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA, USA) and cultured in either EGM (Clonetics, San Diego, CA, USA) or in M199 (GibcoBRL, Life Technologies, Paisley, UK) supplemented with 10% FCS (HyClone Laboratories Inc., Logan, UT, USA)), 2 mM L-glutamine (GibcoBRL), 20 U/ml heparin (GibcoBRL), 10 μg/ml endothelial cell growth factor (Boehringer Mannheim, Mannheim, Germany), 4 μg/ml gentamycin (GibcoBRL) and 1 mM sodium pyruvate (GibcoBRL) (20). They were used within the first 5 passages. Human dermal microvascular endothelial cells (hDMVEC) were obtained from Clonetics and cultured in EGM-MV (Clonetics). U937 (a human hystiocytic lymphoma cell line, ATCC) were cultured in RPMI-1640 (GibcoBRL) supplemented with 10% FCS, 2 mM L-glutamine, 4 μg/ml gentamycin and 1mM sodium pyruvate.

Reagents. Dimethylfumarate (DMF), monoethylfumarate (MEF), fumaric acid (FAcid), phorbol methyl ester (PMA) and lipopolysaccharide (LPS) were purchased from Sigma (St.Louis, MO, USA). Tumor necrosis factor alpha (TNFα), interleukin-4 (IL-4) and interleukin-1 alpha (IL-1α) were obtained from Genzyme (Cambridge, MA, USA). Anti-ICAM-1 monoclonal antibody was purchased from Bender Medsystems (Vienna, Austria), anti-VCAM-1 from Genzyme and anti-E-selectin from R&D Systems (Abingdon, UK). Normal goat serum was obtained from DakoPatts (Glostrup, Denmark), a secondary horseradish peroxidase-labeled goat anti-mouse Ab from Amersham (Buckinghamshire, England). All the reagents for Northern blotting are from Boehringer Mannheim. The DIG-labeled probes were prepared using a random primed labeling method (according to manufacturer's instructions). The VCAM-1 probe is a 3030 bp Xho-fragment of the human cDNA, the E-selectin probe is a 2096 bp XbaI-fragment of the human cDNA, the ICAM-1 probe is a 1880 bp XbaI-fragment of the human cDNA. We used a human acidic ribosomal protein probe as control housekeeping gene. All probes were obtained from R&D Systems.

ELISA. HUVEC were seeded at 20,000/well in 96-well gelatine-coated (0.2% w/v) tissue culture plates (Nunc, Roskilde, Denmark). Confluent HUVEC received fresh medium plus pharmacological agents 1 h prior to stimulation. TNFα was added at 1 ng/ml, IL-4 and PMA at 25 ng/ml, IL-1α at 20 U/ml and LPS at 250 ng/ml. Adhesion molecule expression levels were determined after 4 h for E-selectin and 16 h for ICAM-1 and VCAM-1. Cell monolayers were washed 5 times with PBS, fixed for 15 min in 2% paraformaldehyde and blocked with 2% normal goat serum (NGS). After blocking, a primary mouse anti-human mAb (50 ng/ml anti-ICAM-1, 250 ng/ml anti-VCAM-1 and 1 μg/ml anti-E-selectin in 1% BSA/PBS solution) was incubated for 3 h at room temperature or overnight at 4°C. The monolayers were washed 5 times, blocked and incubated for 1 h with a HRP-conjugated goat anti-mouse antibody (Amersham, Buckinghamshire, England) diluted in 1% NGS/1% BSA/PBS. The amount of cell adhesion molecules was detected through a colorimetric method, using phosphate-citrate buffer with urea hydrogen peroxide tablets (Sigma, St.Louis, MO, USA) and ortho-phenylenediamine dihydrochloride (Sigma) as substrates. The reaction was stopped by adding 2 N sulfuric acid and the absorbance measured on an ELISA plate reader at 490 nm-650 nm. In a typical cell ELISA-experiment quadruplicate plates are accompanied by an MTT-plate (method see below) to assess effects of treatment on cell viability. Cell ELISA values are expressed as $OD_{490\text{-}650nm}/OD_{540\text{-}650nm}$ of the accompanying well in the MTT test.

Cell viability. Cell viability was assessed with a mitochondrial tetrazolium assay (MTT) (21). Briefly, HUVEC were treated under the same conditions as described for the cell ELISA experiments. After the incubation 25 μl/well of an MTT-solution [5 mg/ml MTT (Sigma, St. Louis, MA) in Ca^{++}/Mg^{++}-free PBS] was added and incubated for 3 h. The medium was aspirated and replaced with 100 μl/well isopropanol. The plates were shaken for 15 min and the OD measured at 540 nm-650 nm. In a similar way, the effects of treatments after longer incubation periods (72 h) on viability of confluent HUVEC was assayed. In these experiments the cells received fresh medium with components at the second day.

Cell adhesion assay. U937 were labeled with calcein-acetoxymethylester (Molecular Probes, Eugene, OR, USA) and adherence to a HUVEC monolayer was measured (22). Briefly, cells were pelleted, washed 2 times with warm PBS, resuspended in RPMI-1640 without serum and labeled with 20 μM calcein-acetoxymethylester for 30 min at 37°C. Excess label was removed by washing the cells twice in icecold RPMI-1640 plus 10% FCS. The labeled U937 cells were resuspended in warm RPMI-1640. HUVEC cells were washed in RPMI-1640 and subsequently U937 cells were allowed to adhere to the endothelial monolayer for 90 min at 37°C. After the incubation, the 'total' fluorescence was determined using a Fluoroskan II (Labsystems, Helsinki, Finland) ($\lambda_{excitation}$: 485 nm; $\lambda_{emission}$: 538nm). Nonadherent cells were removed through extensive washing of the plates with warm RPMI-1640 and the 'adherent' fluorescence was determined. The percentage of cells bound to the endothelial monolayer was expressed as ('adherent' fluorescence/'total' fluorescence) $\times$ 100.

RNA isolation and Northern blot. HUVEC, grown in uncoated 6 cm diameter petridishes (Falcon, Becton Dickinson, New Jersey, USA) to confluency, were pretreated with reagents for 1 h and incubated 3 h with TNFα (1 ng/ml). After the incubation, the RNA was isolated, using acid guanidinium thiocyanate-phenol-chloroform (23). Total RNA was size-fractionated on a 1% agarose gel, transferred to a nylon membrane by vacuum blotting and covalently linked by ultraviolet radiation using a Stratlinker 2400 UV crosslinker (Stratagene, La Jolla, CA, USA). Prehybridizations and hybridizations were performed at 50°C in DIG Easy Hyb (Boehringer Mannheim). We used digoxygenin-labeled probes at a concentration of approximately 20 ng/ml. After hybridization the blot membrane was washed with a final stringency of 0.1× SSC (20× SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.4), 0.1% SDS at 68°C. The hybridized probes were immunodetected with an alkaline phosphatase-linked anti-digoxygenin antibody (Boehringer Mannheim) and visualized with the chemiluminescent substrate CPSD (Boehringer Mannheim, 25 mM disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo-[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate).

RESULTS

The effect of alkylated fumarates on TNFα-induced E-selectin, VCAM-1 and ICAM-1 expression was studied using *in situ* ELISA (figure 1A, 1B, 1C). Treatment of the HUVEC monolayers with dimethylfumarate diminished the expression of VCAM-1, ICAM-1 and E-selectin with IC_{50} values approximating 50 μM. Neither monoethylfumarate nor fumaric acid were active. Solvent controls (dimethylsulfoxide) did not affect adhesion molecule expression in HUVEC.

To exclude cytotoxic effects of the combined treatments, a cell viability assay was performed in parallel with each *in situ* ELISA. Treatments were shown not to affect cell viability of HUVEC in parallel MTT assays at 16 h (data not shown) and 72 h (figure 1D). The same observations were made using human dermal microvascular endothelial cells (data not shown).

DIG-Northern blot analysis was used to assay for

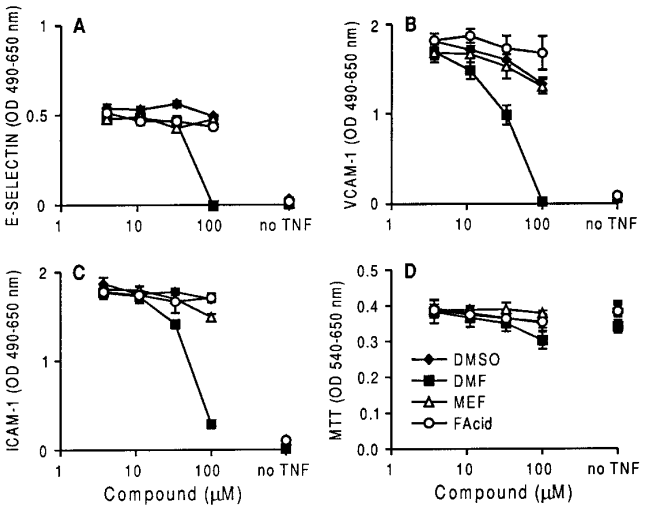

FIG. 1. Inhibitory effects of DMF on TNFα-induced E-selectin (A), VCAM-1 (B) and ICAM-1 (C) expression in HUVEC as measured by *in situ* ELISA 4 h, 16 h and 16 h post-TNFα treatment respectively. The effect of treatments on cell viability in the MTT assay, 72 h after addition of TNFα is displayed in (D). Isolated symbols represent the values for non-cytokine treated HUVEC.

TABLE 1

Spectrum of Inhibitory Activity of 33 μM DMF (VCAM-1 ELISA)

Stimulus (concentration)	Fold induction (VCAM-1)	% inhibition by DMV (33 μM)
TNFα (1 ng/ml)	139 ± 12	78 ± 2
IL-4 (25 ng/ml)	2.7 ± 1.6	83 ± 5
LPS (250 ng/ml)	6.0 ± 2.5	94 ± 10
IL-1α (20 U/ml)	35 ± 3	90 ± 9
PMA (25 ng/ml)	3.5	90
TNFα + IL-4 (*)	390 ± 31	45 ± 6
TNFα + LPS (*)	157 ± 8	74 ± 5
TNFα + IL-1α (*)	111 ± 7	84 ± 1
TNFα ± PMA (*)	13 ± 2	97 ± 3

Note. The table shows the effect of 33 μM DMF on VCAM-1 protein levels in HUVEC, induced by different stimuli, as measured by *in situ* ELISA. (*) Concentration for combinations as in single treatments. "Fold induction" values are calculated as $OD_{490\text{-}650nm}$ after induction/$OD_{490\text{-}650nm}$ without inducing agent. "Percentage inhibition" values are calculated as [$OD_{490\text{-}650nm}$ after treatment with DMF/ $OD_{490\text{-}650nm}$ without treatment (DMSO 0.033%)] × 100. Averages and standard deviations for (n = 3), except for induction with PMA where (n=2).

possible effects of alkylated fumarates at mRNA levels. Figure 2 shows that VCAM-1, ICAM-1 and E-selectin were induced following stimulation with TNFα. DMF reduced all three mRNA levels dose dependently. MEF and FAcid had no effect at the mRNA level. There was no effect on the control housekeeping gene human acidic ribosomal protein.

To assay for the target of action of DMF, inducing agents other than TNFα were tested for VCAM-1 elicitation. TNFα (1 ng/ml) and IL-1α (20 U/ml) were strong and LPS (25 ng/ml) was a moderate inducer of VCAM-1. One ng/ml TNFα plus 25 ng/ml IL-4 was the most potent combination. Table 1 shows that treatment of HUVEC with 33 μM DMF reduced VCAM-1 expression irrespective of the inducing agent or combinations used.

Finally, we tested whether the alkylated fumarate analogues were capable of inhibiting adhesion of U937 to TNFα plus IL-4 stimulated HUVEC (figure 3). Ten % of U937 cells bound to nonstimulated HUVEC whereas after stimulation with 5 ng/ml TNFα plus 5 ng/ml IL-4 approximately 70% adherence was observed. Hundred

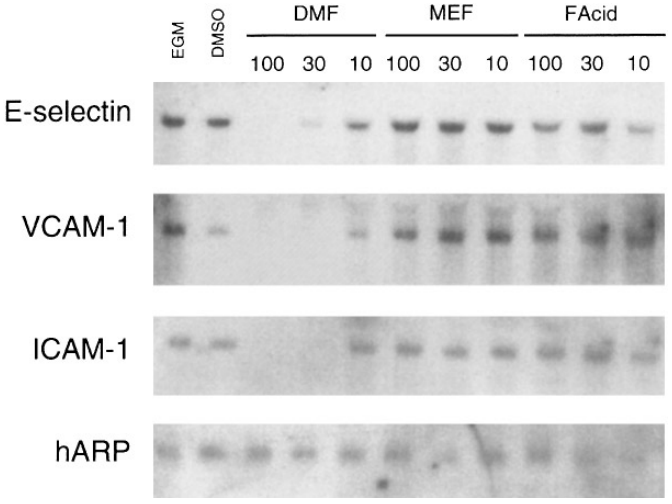

FIG. 2. Effects of different concentrations of alkylated fumarates (expressed in μM) on E-selectin, VCAM-1, ICAM-1 and hARP (a control housekeeping gene) mRNA level, as measured with Northern blot analysis in HUVEC. Total RNA is isolated 3 h post-TNFα induction.

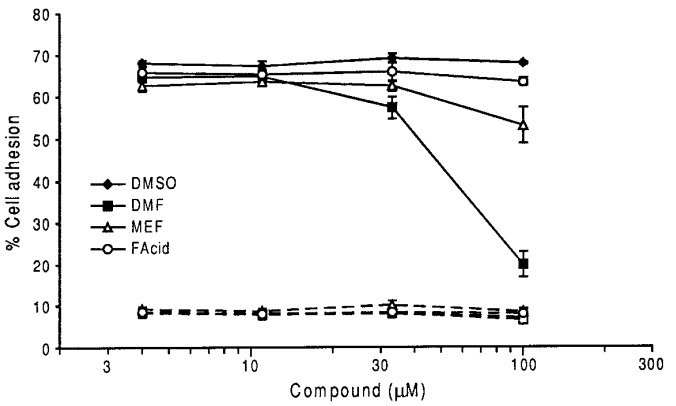

FIG. 3. Adhesion of U937 cells to HUVEC treated with 5 ng/ml TNFα plus 5 ng/ml IL-4 for 18 h (lines) and adhesion of U937 to non-stimulated HUVEC (dashed lines). Data represent percentage adherent cells (see Material and Methods).

μM DMF inhibited the adhesion of U937 cells to stimulated HUVEC by 70%. Monoethylfumarate only showed a minor effect at 100 μM, whereas fumaric acid did not interfere with adherence at all.

DISCUSSION

Psoriasis is a chronic inflammatory skin disease with prominent epidermal, dermal, vascular and immune involvement (24-26). Effective therapies for psoriasis may affect either the immune (e.g. cyclosporin), the epidermal (e.g. retinoids) or both aspects of the disease (e.g. methotrexate). For alkylated fumarates - the active components of a marketed anti-psoriaticum Fumaderm - most of the studies concerning mode of action are focussed on their antiproliferative effect in keratinocytes (17,18). Although vascular activation is a major component in the pathophysiology of psoriasis, the effect of active agents on vascular cells activated *in vitro* is poorly studied (27). We investigated the effect of dimethylfumarate on endothelial cells, more specifically on cytokine-induced adhesion molecule expression.

We here demonstrate that DMF, but not MEF or FAcid, inhibits cytokine-induced expression of VCAM-1, ICAM-1 and E-selectin mRNA and protein in HUVEC. This inhibition leads to a reduced adhesion of U937 cells to a HUVEC monolayer, activated with the most potent combination of cytokines, TNFα plus IL-4, underscoring the functional relevance of this inhibitory effect in terms of cell-recruiting capabilities of endothelial cells *in vitro*.

The fact that DMF reduces VCAM-1 expression elicited by TNFα as well as by a variety of other inducing agents, is interesting from a mechanistic point of view. It critically suggests that DMF does not interfere with one specific cytokine signaling cascade but rather downstream at the level where these cascades converge. On the other hand, confirmation of DMF's inhibitory effects on VCAM-1, ICAM-1 and E-selectin mRNA levels implies targets of action upstream transcription. *De novo* expression of these adhesion molecules relies on the activation of a cytokine-inducible enhancer in the promotor of their genes (1). These enhancers are unique for each adhesion molecule gene, but all three contain a nuclear factor-κB responsive element. In non-activated endothelial cells, the transcription factor NF-κB is sequestered in the cytosol through association with an inhibitory factor IκB (28). One of a variety of signaling cascades which were shown to release and activate NF-κB by degradation of IκB is of particular relevance to the target of action of DMF. Cytokine-induced shifts in intracellular thiol levels towards low reduced glutathione and/or high reactive oxygen levels seem to be critical for NF-κB activation (29). Dimethylfumarate is capable of inducing enzymes responsible for the synthesis and the maintenance of gluthatione pools (30,31) resulting in increased intracellular thiol levels in H9 cutaneous lymphoma T cells (30), in a monocytoid U1 cell line (32), and in porcine aortic endothelial cells (33). Gel mobility shift assays confirm that DMF abrogates PMA-induced NF-κB activation in U1 cells (32) and arsenite-induced NF-κB translocation in aortic endothelial cells (33). From a different point of view, we observed physiological effects of DMF on cytokine-induced adhesion molecule expression in endothelial cells (IC_{50} approximating 50 μM). We conclude that from a mechanistic point of view, our data are in agreement with recently made observations of Prochaska *et al.*(32) and Barchowsky *et al.*(33).

In summary, we show that dimethylfumarate but not monoethylfumarate or fumaric acid affects another cell type - besides keratinocytes - implicated in inflammatory dermatoses, such as psoriasis. Cytokine-induced cell adhesion molecule expression on endothelial cells is significantly inhibited by DMF and leads to a reduced adhesion of the monocyte cell line U937 to activated HUVEC. As the presence of inflammatory cells in dermis and epidermis is an important feature in psoriasis, the endothelial cell might represent another target in the observed anti-psoriatic activity of dimethylfumarate.

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