Inhibition of Dendritic Cell Differentiation by Fumaric Acid Esters

Kejian Zhu1 and Ulrich Mrowietz
Department of Dermatology, University of Kiel, Kiel, Germany

Fumaric acid esters have proved to be effective for the systemic treatment of severe psoriasis vulgaris. These compounds have been shown to induce a Th2-like cytokine secretion pattern in T cells and to reduce keratinocyte proliferation in vitro. Dendritic cells seem to be of major importance as regulatory cells driving the psoriatic tissue reaction. Monocytes or CD34-positive myeloid progenitor cells are precursors of dendritic cells that can be generated in vitro by culture with granulocytemacrophage colony-stimulating factor and interleukin-4. Using this model the effect of fumaric acid esters on granulocyte-macrophage colony-stimulating factor/interleukin-4-induced differentiation of monocyte-derived dendritic cells was investigated. The results of this study show that dimethylfumarate as well as methylhydrogenfumarate-calcium-salt (0.01–100 μg per ml) concentration-dependently inhibit monocyte-derived dendritic cell differentiation. This was reflected by an inhibition of CD1a, CD40, CD80, CD86, and HLA-DR expression as well as by a reduced capacity of dimethylfumarate-treated monocyte-derived dendritic cells to stimulate lymphocytes in the allogeneic mixed lymphocyte reaction. Other fumaric acid esters showed no effect on monocyte-derived dendritic cell-differentiation. At higher concentrations (30–100 μg per ml) dimethylfumarate, but not methylhydrogenfumarate calcium-salt induced apoptosis in monocyte-derived dendritic cells as measured by expression of Apo 2.7 and DNA fragmentation (TUNEL assay). These data point to a high susceptibility of the monocyte/dendritic cell system to dimethylfumarate and its main metabolite methylhydrogenfumarate. Other fumaric acid esters investigated were without effect. As the effects of fumarates on monocyte-derived dendritic cells observed occur at concentrations 20-fold lower compared with lymphocytes, our data seem to be of relevance in explaining the possible mode of action of these compounds in psoriasis. Key words: apoptosis/dendritic cells/fumarate/psoriasis/treatment. J Invest Dermatol 116:203–208, 2001

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Reprint requests to: Dr. Ulrich Mrowietz, Department of Dermatology, University of Kiel, Schittenhelmstr. 7, 24105 Kiel, Germany.
Email: umrowietz@dermatology.uni-kiel.de

Abbreviations: Ca-MHF methylhydrogenfumarate calcium-salt; DMF, dimethylfumarate; EC50, half-maximal effective dose; FAE, fumaric acid esters; MHF, methylhydrogenfumarate; TUNEL terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling

1Present address: Department of Dermatology, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, People’s Republic of China.
beneficial effect of DMF as the major ingredient of the marketed drug and its main metabolite MHF in the treatment of psoriasis. Additional experiments performed in order to investigate the underlying mechanisms of this effect more closely point towards the induction of apoptosis in dendritic cells by DMF.

MATERIALS AND METHODS

Culture medium and reagents The standard medium for cell culture was RPMI 1640 (BioConcept, Umkirch, Germany) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (BioConcept, Umkirch, Germany), 100 U penicillin per ml, 100 μg streptomycin per ml. For dendritic cell culture 100 μg granulocyte-macrophage colony-stimulating factor (GM-CSF) per ml (Leucomax, Novartis Pharma, Nürnberg, Germany) and 10 ng IL-4 per ml (kindly supplied by Schering-Plough, Kenilworth, Nj) was added to the supplemented medium.

Phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies against the epitopes CD1a, CD14, CD40, CD80, CD86, and HLA-DR as well as their respective isotype controls were from Coulter-Immunotech (Krefeld, Germany).

The following fumaric acid esters (Merck, Darmstadt, Germany) were used in this study: methylhydrogenfumurate (MHF), methylhydrogenfumurate calcium salt (Ca-MHF), ethylhydrogenfumurate magnesium salt, ethylhydrogenfumurate calcium salt, DMF, fumaric acid, monoethylfumurate, pentylhydrogenfumurate, and isopropylhydrogenfumurate. DMF was dissolved in dimethyl sulfoxide and later diluted with standard medium, the other FAE used were dissolved in standard medium. Dilutions of FAE were prepared freshly and sterilized by filtration through 0.2 μM pore-size membranes (Sartorius, Göttingen, Germany).

Except for DMF and Ca-MHF, FAE were used at a concentration of 10 μg per ml. DMF and Ca-MHF were employed at various concentrations ranging from 0.01 to 10 μg per ml. Standard medium and DMSO were equally tested as solvent control.

Generation of dendritic cells and cell culture Human purified monocytes were isolated by counterflow centrifugation elutriation from peripheral blood mononuclear cells, obtained by Ficoll Paque (Biochrom, Berlin, Germany) density gradient centrifugation of ethylenediamine tetraacetic acid-anticoagulated venous blood from healthy donors after informed consent. Purity of monocytes was assessed by cell-size analysis (Coulter counter-channelizer), and by microscopic evaluation of Giemsa-stained cytopsin preparations.

Monocytes were cultured at a density of 1.5–2 × 10⁶ per ml in six-well flat bottom tissue-culture plates (Becton-Dickinson, Heidelberg, Germany) at 37°C in a humidified atmosphere with 5% CO₂ in standard medium with GM-CSF/IL-4 to generate immature, inflammatory-type monocyte-derived dendritic cells (Sallusto and Lanzavecchia, 1994). Monocytes were treated from the beginning with FAE or DMSO (final concentration 0.1%) or left with medium alone. Differentiation of dendritic cells was controlled daily using an inverted microscope.

Flow cytometric analysis GM-CSF/IL-4-induced differentiation of monocyte-derived dendritic cells and the effect of FAE on this process was assessed by single color flow cytometry. Analysis was performed using an EPICS-XL flow cytometer and system II software (Coulter). Briefly, cells were harvested after 5 d of culture and resuspended in phosphate-buffered saline (PBS). Pelleted cells (5 × 10⁶) in 100 μl PBS were incubated for 30 min at room temperature in darkness with antibodies against CD1a, CD14, CD40, CD80, CD86, and HLA-DR as well as their corresponding isotype control antibodies to determine the level of background staining. At least 2 × 10⁶ viable monocyte-derived dendritic cells per sample, gated according to forward and side scatter characteristics, were analyzed.

Allogeneic mixed lymphocyte reaction (MLR) MLR was performed using purified allogeneic T cells as responder cells and monocyte-derived dendritic cells as stimulator cells. Purified T cells were obtained by negative selection using the MACS-system (Miltenyi Biotech, Bergisch Gladbach, Germany) employing monoclonal antibodies coupled to magnetic beads against the following epitopes: CD11b, CD16, CD19, CD36, and CD56. Briefly, 2 × 10⁵ monocyte-derived dendritic cells after 5 d of culture in the presence of DMF (1.0 and 10 μg per ml) or DMSO (0.01%, vol/vol) as solvent control were mixed with 5 × 10⁵ responder cells in a final volume of 200 μl RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 mM L-glutamine, 100 U penicillin per ml, 100 μg streptomycin per ml and 10 mM HEPES in 96-well round-bottomed microtiter plates (Costar, Bodenheim, Germany) at 37°C in a humified atmosphere with 5% CO₂ for 5 d. Three different monocline-derived dendritic cells/T cell ratios were investigated: 1:1, 1:10, and 1:100. Proliferation of lymphocytes was assessed after addition of 1 μCi per well of [³H]thymidine (specific activity: 5 Ci per mmol; Amersham, Braunschweig, Germany) for the last 24 h of culture. Cells were harvested on glass fiber with a semiautomated cell harvester apparatus (Skatron, Vienna, Austria) and [³H]thymidine incorporation was measured in a liquid scintillation spectrometer (Beckman, Munich, Germany). All experiments were performed in triplicate and expressed as mean counts per minute (cpm) ± SD.

Determination of apoptosis by flow cytometry Flow cytometry was used for detecting the cells undergoing apoptosis. Monocyte-derived dendritic cells after 5 d of culture in the presence of DMSO or FAE were harvested and washed twice with PBS. Then, 1 × 10⁵ cells suspended in 100 μl of PBS were incubated for 30 min at room temperature in darkness with a monoclonal antibody against Apo 2.7 (Coulter-Immunotech). To exclude nonspecific staining, cells stained with corresponding isotype-matched monoclonal antibodies were used as controls. For each sample, a minimum of 10,000 cells, gated according to the forward and side scatter characteristics, were analyzed.

Fluorescence-activated cell sorting (FACS) analysis FACS was performed using a FACScan (Becton Dickinson, Mountain View, CA). Analysis was performed from the data obtained from the unpaired Student’s t test. A p < 0.05 was considered significant.

RESULTS

Viability and purity of monocytes and monocyte-derived dendritic cells Human monocytes showed a purity of greater than 95% and a viability of >98% after elutriation. After 5 d of culture with GM-CSF/IL-4 the viability of monocyte-derived dendritic cells was greater than 90% as judged by Trypan blue exclusion.

Effect of FAE on the differentiation of monocyte-derived dendritic cells Differentiation of monocyte-derived dendritic cells by culturing monocytes for 5 d in standard medium with GM-CSF/IL-4 was analyzed by flow cytometry using monoclonal antibodies against CD1a, CD14, CD40, CD80, CD86, and HLA-DR. The results shown in Fig 1 demonstrate the typical CD1a/CD40 (high), CD14 (low) monocyte-derived dendritic cells phenotype as compared with monocytes. CD80, CD86, and HLA-DR were also expressed on monocyte-derived dendritic cells.

Treatment of monocytes with DMF (10 μg per ml), (Fig 1A) a concentration not inducing significant apoptosis (see below and Fig 3) lead to a significant decrease in cells expressing CD1a, CD40, CD86, and HLA-DR. There was no change in CD14 expression by DMF. DMSO (0.01%, vol/vol) corresponding to a FAE concentration of 10 μg per ml had no effect on the differentiation of monocyte-derived dendritic cells. Figure 1(B) demonstrate the effect of Ca-MHF (100 μg per ml) on MoDC differentiation. The substance reduced expression of CD1a, CD40, CD80, CD86, and HLA-DR significantly. Interestingly, CD14 expression was upregulated by Ca-MHF at this concentration, indicating a pronounced shift of the cells to a monocyte/macrophage phenotype. Furthermore, all other FAE investigated
as well as fumaric acid had no effect on monocyte-derived dendritic cells differentiation (data not shown).

Concentration-dependent inhibitory effect of DMF and Ca-MHF on the differentiation of monocyte-derived dendritic cells

We next analyzed if the effect of DMF on monocyte-derived dendritic cells differentiation was concentration related. For comparison, the main DMF metabolite MHF in the form of calcium salt was tested equally. Figure 2 demonstrates concentration-dependent effects of DMF and Ca-MHF (0.01–100 μg per ml) on CD1a, CD14 expression and HLA-DR expression. A significant inhibition of CD1a expression was found for DMF at a concentration of 10 μg per ml (p < 0.01) and 100 μg per ml (p < 0.001), respectively. Expression of HLA-DR was significantly reduced at all concentrations used (p < 0.05).

Ca-MHF significantly inhibited CD1a-expression at 1 and 10 μg per ml (p < 0.05) and 100 μg per ml (p < 0.001), respectively. At a concentration of 10 and 100 μg per ml expression of HLA-DR was also reduced (p < 0.05). Ca-MHF, but not DMF, significantly up-regulated CD14 at 100 μg per ml (p < 0.05).

DMF inhibit the antigen-presenting function of monocyte-derived dendritic cells

Monocyte-derived dendritic cells function as potent antigen-presenting cells and possess a high capacity to stimulate lymphocytes in the MLR. As phenotypic analysis showed that DMF inhibits differentiation (CD1a/CD40-low) we analyzed the stimulatory capacity of DMF-treated monocyte-derived dendritic cells in allogeneic MLR.

In comparison with solvent (DMSO)-treated control cells [3H]thymidine uptake of purified allogeneic T cells was decreased in a concentration-dependent fashion after incubation with DMF-treated monocyte-derived dendritic cells (Table I). Inhibition of DMF-induced [3H]thymidine uptake was most pronounced using monocyte-derived dendritic cells/T cell ratios of 1:1 and 1:10 in comparison with the solvent control.

Induction of apoptosis by FAE

As DMF and Ca-MHF in concentrations above 50 μg per ml caused increased granularity, reduced dendrites and decreased viability we asked whether these morphologic alterations could be due to the induction of apoptosis. To ensure a safe detection of apoptotic processes two independent assay systems were employed.
As shown in Fig 3(A) DMF not Ca-MHF, however, induced a concentration-dependent increase in the number of apoptotic cells as determined by Apo 2.7 expression. At the highest concentration of DMF used (100 μg per ml) more than 90% of monocyte-derived dendritic cells stained positive for Apo 2.7 indicating induction of apoptosis.

Using TUNEL as a second method to detect apoptosis DMF was shown to induce DNA fragmentation in a concentration-dependent manner (Fig 3B). At 100 μg per ml DMF about 80% of monocyte-derived dendritic cells were demonstrated to be apoptotic by terminal deoxynucleotidyl transferase labeling. Ca-MHF-treated monocyte-derived dendritic cells showed no terminal deoxynucleotidyl transferase labeling, indicating DNA integrity.

**DISCUSSION**

Fumaric acid esters have been found to be effective in the treatment of psoriasis by empiric means (Schweckendiek, 1959). Meanwhile, fumarates have successfully been used for more than 40 y, mainly in Germany and in the Netherlands as a defined mixture of different FAE, with DMF being the major compound. DMF is rapidly hydrolyzed into MHF (also called monomethylfumarate), which is regarded to be a major active metabolite. On the basis of the chemical properties of DMF, this compound may penetrate cellular membranes more easily as compared with Ca-MHF or MHF.

Several studies have been conducted to elucidate the mechanism of action of FAE in the treatment of psoriasis and the results of these investigations have been summarized recently (Mrowietz et al., 1999). MHF has been shown to stimulate tumor necrosis factor-α, IL-10, and IL-1 receptor antagonist production in human peripheral blood mononuclear cells without an effect on IL-12 secretion (Asadullah et al., 1997). Besides an antiproliferative effect on keratinocytes (Sebök et al., 1994) and lymphocytes (Petres et al., 1975) in vitro, cells of the monocytic lineage seem to be an additional important target for the action of FAE.

The results of our study show that DMF and its main in vivo metabolite MHF as investigated by the use of its calcium-salt is able to interfere with the differentiation of dendritic cells from monocytes in vitro. DMF and MHF inhibited upregulation of the
The TUNEL technique demonstrates DNA apoptosis in monocyte-derived dendritic cells, whereas Ca-MHF could be shown that DMF concentration-dependently induced fragmentation (Loo and Rillema, 1998). Using both methods it was observed at higher concentrations of FAE (> 50 mg per ml) compared with medium control or solvent (DMSO) control (maximum concentration 0.1%, vol/vol) as measured by the expression of Apo 2.7 (A) or by TUNEL (B). Mean and SD, n = 5.

As compared with other in vitro effects of FAE, such as the induction of IL-4/IL-5 in MHF-treated T cells, the effect of DMF and Ca-MHF on monocyte-derived dendritic cells differentiation was observed at a 20–200 times lower concentration (Mrowietz et al., 1999).

To address the question if changes in cellular morphology observed at higher concentrations of FAE (> 50 μg per ml) may be due to the induction of apoptosis, we performed additional experiments with DMF and Ca-MHF in concentrations up to 100 μg per ml. Apoptosis was measured by two independent methods. The expression of the marker Apo 2.7 is a sensitive measure to detect induction of apoptosis at an early stage (Zhang et al., 1996). The TUNEL technique demonstrates DNA fragmentation (Loo and Rillema, 1998). Using both methods it could be shown that DMF concentration-dependently induced apoptosis in monocyte-derived dendritic cells, whereas Ca-MHF had no effect (Fig 3). From these results it may be concluded, that the inhibition of monocyte differentiation by DMF at higher concentrations may be due to the induction of apoptosis.

Dendritic cells are regarded to play a crucial part in the pathogenesis of psoriasis. These cells are increased in number in lesional skin and show enhanced immunologic activity (Nestle et al., 1993, 1994). In the light of recent evidence that psoriasis may be caused by (auto)antigen(s) dendritic antigen-presenting cells will drive the pathogenetic process (Valdimarsson et al., 1997; Drakemith et al., 2000). Therefore, targeting the dendritic cell system seems to be an effective measure to treat psoriasis. As dendritic cells do not proliferate in situ, precursors such as monocytes or CD34+ myeloid progenitor cells are recruited from the peripheral blood differentiating in the tissue into immunologically active cells (Müller and Randolph, 1999). GM-CSF/IL-4-induced monocyte-derived dendritic cells represent so-called immature dendritic cells, which home at sites of inflammation as recently demonstrated (Barratt-Boyes et al., 1998). Inhibition of monocyte-derived dendritic cell differentiation, therefore, may lead to a decreased immunologic activity as we have shown in this study.

Recently, IL-10 was found to have beneficial effects in patients with severe psoriasis (Asadullah et al., 1998; Reich et al., 1998). As demonstrated earlier IL-10 inhibited GM-CSF/IL-13-induced differentiation of human monocytes into monocyte-derived dendritic cells (Allavena et al., 1998). A reduction of CD1a expression and an upregulation of CD14 as well as a reduced ability of monocyte-derived dendritic cells to stimulate allogeneic T cells in the MLR was found after IL-10 treatment. Further analysis revealed, that IL-10 shifted GM-CSF/IL-13-induced dendritic cell development towards a macrophage-like differentiation. Very recently, 1,25-dihydroxycholecalciferol, the active metabolite of vitamin D3, was found to inhibit monocyte-derived dendritic cell differentiation and T cell-stimulating capacity in the MLR (Penna and Adorni, 2000; Piemonti et al., 2000). This, however, was not described to be due to the induction of apoptosis.

The loss of antigen-presenting activity of monocyte-derived dendritic cells as shown by the decreased ability to stimulate the MLR after DMF treatment led to a decreased immunologic activity. The inhibition of CD40 expression and the down-regulation of CD80/CD86 may be directly linked to this effect (Grewal and Flavell, 1998; van Kooten and Banchereau, 2000). As this is achieved at a 20-fold lower concentration as compared with other FAE at these concentrations did not induce apoptosis. Further investigations are needed in order to define more closely the effects of FAE observed in this study with regard to the clinically proven effectiveness of the compounds in the treatment of severe psoriasis.
REFERENCES


