Dimethylfumarate Inhibits Tumor-Necrosis-Factor-Induced CD62E Expression in an NF-κB-Dependent Manner

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Fumaric acid esters are thought to improve psoriasis by altering leukocyte, keratinocyte, and/or endothelial functions. To determine specificity, kinetics, and molecular mechanisms of different fumaric acid esters in their ability to inhibit endothelial cell activation, we analyzed CD62E and CD54 expression in endothelial cells *in vivo* and *in vitro*. In lesional skin of psoriatic patients, oral fumaric acid ester treatment resulted in a marked reduction of CD62E but not CD54 expression on dermal microvessels. Using human umbilical vein endothelial cells, dimethylfumarate almost completely inhibited tumor-necrosis-factor-induced CD62E, but not CD54 expression at concentrations ≦70 μM, mimicking the situation *in vivo*. A 60 min dimethylfumarate preincubation was sufficient to block tumor-necrosis-factor-induced CD62E expression for up to 24 h. In contrast, equimolar concentrations of methylhydrogenfumarate, the hydrolysis product of dimethylfumarate, did not suppress tumor-necrosis-factor-induced CD62E expression. Likewise, all fumaric acid esters other than dimethylfumarate were ineffective. Using CD62E, NF-κB, or AP-1-responsive promoter constructs, dimethylfumarate inhibited tumor-necrosis-factor-induced activation of the CD62E and the NF-κB but not the AP-1 promoter construct. In summary, at a dose range ≦70 μM, dimethylfumarate appeared to be a specific inhibitor of CD62E expression in an NF-κB-dependent manner.* ***Key words: CD54/endothelial cells/fumaric acid esters/methylhydrogenfumarate/psoriasis. J Invest Dermatol 117:1363–1368, 2001***

Fumaric acid esters (FAE) have been used empirically in the treatment of psoriasis for many years (reviewed by Mrowietz *et al*, 1999). In the first controlled study, a therapeutic potency was shown for dimethylfumarate (DMF) only and not for salts of ethylhydrogenfumarates (EHF) (Nieboer *et al*, 1989). Kolbach *et al* used a combination of DMF plus EHF salts and found this mixture superior to DMF alone (Kolbach and Nieboer, 1992). As all subsequent open and controlled clinical studies used this mixture of FAE (Altmeyer *et al*, 1994; Mrowietz *et al*, 1998), questions regarding the most active ingredient(s) of the FAE mixture and the mode of action are still under debate. The situation is further complicated by the fact that, due to its chemical structure, DMF hydrolyzes into methylhydrogenfumarate (MHF) and methanol, but MHF has yet not been tested in a monotherapy regimen.

In vitro studies have only partially resolved the question of individual efficacies of different FAE. For example, in lymphocytes, MHF modulated cytokine expression towards a Th2 cytokine profile (de Jong *et al*, 1996; Asadullah *et al*, 1997). Subsequent work employing a different setup confirmed and extended these data by showing that also DMF switched T cell responses towards a Th2 cytokine profile (Ockenfels *et al*, 1998). Likewise, in keratinocytes, both MHF and DMF inhibited keratinocyte proliferation (Thio *et al*, 1994). Both MHF and DMF inhibited monocyte differentiation into dendritic cells (Zhu and Mrowietz, 2001). In vascular endothelial cells, DMF but not EHF suppressed tumor necrosis factor (TNF) induced endothelial adhesion molecule expression, but MHF has not yet been tested (Vandermeeren *et al*, 1997).

We therefore analyzed the effect of different FAE on CD62E and CD54 expression in vascular endothelial cells *in vivo* and *in vitro*. CD62E and CD54 are inducible endothelial adhesion molecules. They play a very important role in mechanisms governing tethering, rolling, and tight adhesion of leukocytes on endothelial cell surfaces. This cascade of events is required to initiate and perpetuate tissue inflammation (Bevilacqua *et al*, 1989; Lawrence *et al*, 1995). Here, we show that DMF, but not MHF, at concentrations ≦70 μM, is a specific inhibitor of CD62E expression in an NF-κB-dependent manner.

MATERIALS AND METHODS

Cell culture and reagents Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords and cultured as described previously (Petzelbauer *et al*, 1993). HUVEC were grown in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Gaithersburg, MD) containing 20% heat-inactivated fetal bovine serum (Life

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Abbreviations: CaMF, calcium bis(monomethylfumarate); DMF, dimethylfumarate; EHF, ethylhydrogenfumarate; FA, fumaric acid; FAE, fumaric acid esters; HUVEC, human umbilical vein endothelial cells; MHF, methylhydrogenfumarate.

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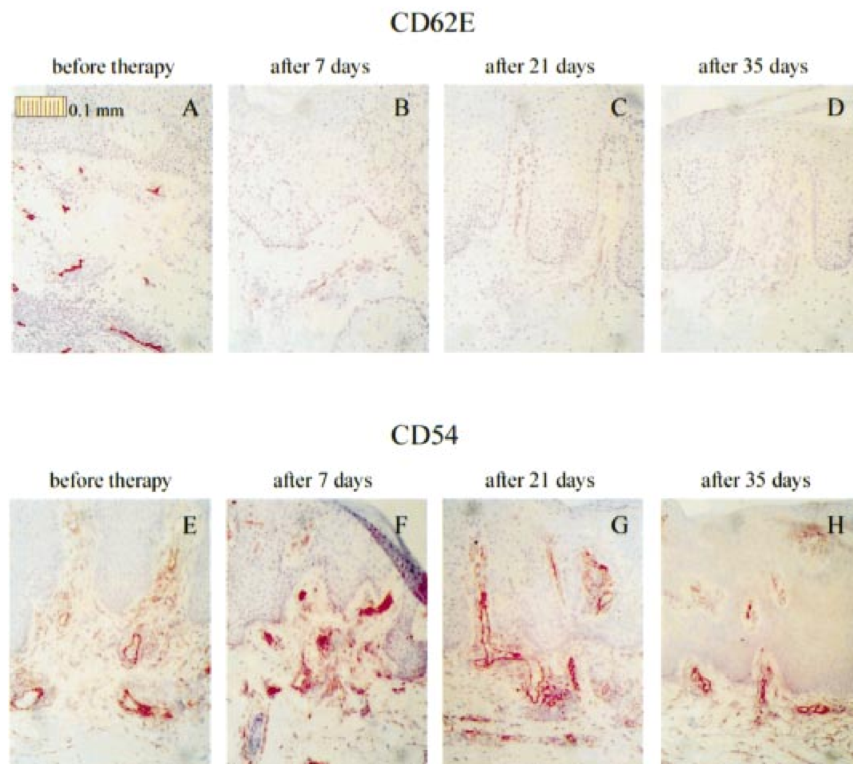


Figure 1. Fumaric acid derivatives inhibit CD62E expression *in vivo*. Immunohistochemistry of psoriatic skin lesions. Five millimeter punch biopsies were obtained before and after 7, 21, and 35 d of therapy with FAE (Fumaderm). The daily DMF dosages were 60 mg DMF and 150 mg EHF (Ca^{2+}, Mg^{2+}, and Zn^{2+} salts) between days 1–7, 120 mg DMF and 95 mg EHF salts between days 8–21, and 360 mg DMF and 285 mg EHF salts between days 22 and 35. Serial sections of the specimens were stained with CD62E (*A–D*) and CD54 (*E–H*).

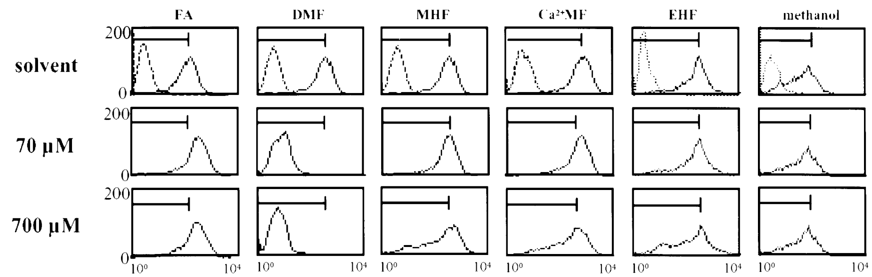
Technologies), 2 mM per l glutamine (Life Technologies), 50 IU per ml penicillin–streptomycin (Life Technologies), and endothelial cell growth supplement with heparin (Promocell, Heidelberg, Germany). Cells were used between passages 2 and 6. TNF-α was purchased from Strathmann Biotech (Hamburg, Germany) and used at a concentration of 10 ng per ml. All FAE were from Fumapharm (Muri, Switzerland). DMF was dissolved in methanol as a 70 mM stock solution. Fumaric acid (FA), calcium bis(monomethylfumarate) (CaMF), MHF, and EHF were prepared as a 70 mM stock solution in IMDM and stored for up to 3 d. Fumaderm (Fumamedica, Herne, Germany) is the only FAE registered for oral treatment of psoriasis. One tablet consists of DMF (120 mg) and a mixture of Ca^{2+}, Mg^{2+}, and Zn^{2+} salts of EHF (95 mg). Maximal daily dose is six tablets.

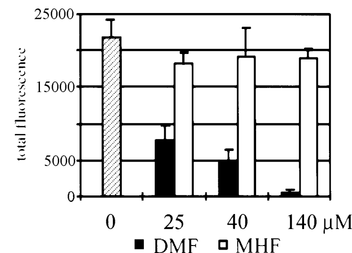
Immunohistochemistry After informed consent, 5 mm punch biopsies from lesional and nonlesional skin of three patients with severe psoriasis before and during systemic therapy with FAE (Fumaderm) were obtained from the Department of Dermatology of the University of Kiel, Germany. The biopsies were snap frozen in liquid nitrogen and shipped on dry ice. Specimens were cut on a Leica cryostat (Vienna, Austria), air dried, and fixed with acetone for 10 min at 4°C. Then, a three-step immunohistochemistry technique was performed using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA) as described previously (Petzelbauer *et al*, 1993). First-step reagents were CD54 (clone BBIG-I1) and CD62E (clone BBIG-E4) (both R&D Systems, Minneapolis, MN). Bound peroxidase was visualized by 3-amino-9-ethylcarbazole (Sigma-Aldrich, Deisenhofen, Germany). Sections were counterstained with Mayers Hemalaun and examined with an Olympus microscope (Olympus Optical, Hamburg, Germany).

Fluorescence-activated cell sorter (FACS) analysis Confluent HUVEC were stimulated with TNF for 4 h in the presence or absence of the indicated FAE. Cells were suspended in trypsin/ethylenediamine tetraacetic acid (Life Technologies), washed in phosphate-buffered saline (PBS), and incubated with CD54, CD62E, or mouse IgG1 κ isotype control (MOPC 31c, Sigma-Aldrich) in PBS (each 1 μg per ml) for 30 min on ice. After additional washings and incubation with the fluorescein isothiocyanate conjugated second-step antimouse IgG antibody (Sigma-Aldrich, 1 μg per ml) for 30 min on ice, surface-bound fluorescence was analyzed by FACScan (Becton Dickinson, San Jose, CA). Total fluorescence values were calculated as described previously (Petzelbauer *et al*, 1993); briefly the absolute number of positive cells was multiplied by the mean channel fluorescence corrected for the individual background. Data were pooled from three or five experiments and the mean ± SD was calculated.

RNA isolation and northern hybridization Total RNA was isolated using the RNEasy Kit from Qiagen (Hilden, Germany). A total of 10 μg RNA was mixed with RNA loading buffer (Sigma-Aldrich) and denatured for 10 min at 65°C prior to separation on a 0.8% agarose gel. RNA was transferred on Nylon membranes and stained with methylene blue. Probes for CD62E and CD54 (a cocktail of six oligonucleotide antisense probes, R&D Systems) were end-labeled with ^{32}P by using T4 polynucleotide kinase. Blots were hybridized with the labeled probes in ExpressHyb buffer (Amersham, Buckinghamshire, U.K.) for 60 min at 42°C, and then washed with 2 × SSC (0.3 M NaCl, 30 mM sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) first, then in 0.5% SSC plus 0.1% SDS two times, and a last wash with 0.25 × SSC plus 0.1% SDS. The blots were then exposed to Kodak films (Eastman Kodak, Rochester, NY) for 1 or 2 d. Northern signals were determined by densitometry (Kodak electrophoresis documentation and analysis system 120) and normalized to their 28S RNA bands.

Reporter assays A triplicate of the consensus AP-1 site (48 bp, 3′-TGTGATGACTCAGGTT) or a triplicate of the consensus NF-κB site (60 bp, 3′-AATCGTGGAATTTCCTCTGA) flanked by SpeI sites were inserted into the SpeI site of the pTK-UBT-luc vector, respectively (de Martin *et al*, 1993). A 1.3 kb construct of the CD62E promoter spanning from bp –1285 to bp +482 was inserted into the NdeI site of the pMAM neo-luc vector (Clontech) as described previously (Cooper *et al*, 1996). Cells were transfected using the standard Ca^{2+} phosphate precipitation method as described elsewhere (Gille *et al*, 1996). Briefly, HUVEC between passages 3 and 5 were seeded in gelatine-coated six-well plates (Falcon) and grown to 70%–80% confluence. For transfection, 2.5 μg of the respective promoter construct was added per well. To control for transfection efficiency, cotransfection with 500 ng of a pSV-β-galactosidase control vector (Promega, Madison, WI) was performed in each experiment. Two days after transfection, cells were stimulated for 2 h with 10 ng per ml TNF with or without 40 μM DMF. Cells were then trypsinized, pelleted, rinsed, and resuspended in 200 μl reporter lysis buffer (Promega) for 15 min according to the manufacturer's instructions. Luciferase activity was measured with a Berthold AutoLumat LB9507 luminometer using the luciferase assay system (Promega). β-Galactosidase activity was determined by a β-galactosidase enzyme assay system (Promega). Luciferase activities obtained with the respective promoter constructs were normalized according to their





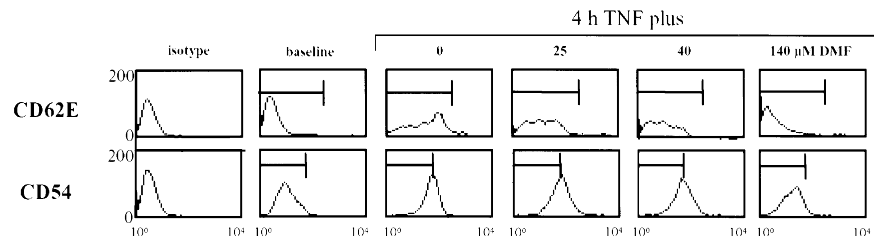


Figure 2. Effects of FAE on CD62E and CD54 expression *in vitro*. (*A*) Flow cytometric analysis of CD62E expression in HUVEC. Cells were TNF-treated without or with the indicated concentrations of FA, FAE, or methanol alone. *Bold lines* represent CD62E expression, *dotted lines* isotype control staining. One representative out of three experiments is shown. (*B*) Summary of three independent experiments. Total fluorescence values are calculated as described in *Materials and Methods*, mean ± SD. *Hatched bar*, TNF-stimulated cells; *closed bars*, DMF plus TNF; *open bars*, MHF plus TNF. (*C*) Flow cytometric analysis comparing CD62E (3 h TNF) and CD54 (18 h TNF) expression at different concentrations of DMF. One representative out of four experiments is shown.

β-galactosidase activity. The variation of β-galactosidase activity within individual experiments was < 10%.

RESULTS

FAE suppress CD62E expression *in vivo* Skin biopsies of psoriatic lesions and normal skin before and at weeks 1, 3, and 5 during oral treatment with FAE (Fumaderm) were analyzed for CD62E and CD54 expression. To allow a semiquantitative evaluation of the staining intensity with the respective antibodies, we performed serial dilutions of monoclonal antibodies. For CD62E, at a concentration of 0.3 μg per ml, minimal reactivity was seen in untreated lesional skin and CD62E staining was undetectable at days 7 through 35 after the initiation of treatment. At 3 μg per ml, strong reactivity was seen at days zero and 7, and staining was undetectable at days 22 and 35. Staining at an antibody concentration of 1 μg per ml is shown in **Fig 1**, showing strong CD62E reactivity at day zero, weak reactivity at day 7, and no reactivity thereafter. For CD54, at a concentration of 0.3 μg per ml, all specimens showed an equally weak staining pattern; at 3 μg per ml all specimens uniformly showed strong staining. Also the CD54 expression on keratinocytes and dermal infiltrating cells persisted. Histologically, lesions still showed a psoriatic phenotype. Clinical improvement was seen at day 21. Centers have cleared at day 35 with active margins still present.

DMF suppresses CD62E expression in cell culture The effects of different FAE and of FA on TNF-induced CD62E expression in HUVEC are shown in **Fig 2(*A*)**. DMF completely blocked the TNF-induced CD62E expression at concentrations ≦70 μM compared with the FA control and with other FAE tested. A less than 10% inhibition of CD62E expression by FAE other than DMF was seen at concentrations of 700 μM. Methanol did not inhibit TNF-induced expression of CD62E at any concentration tested. A summary of three independent experiments comparing DMF and MHF effects on CD62E expression is shown in **Fig 2(*B*)**. **Figure 2(*C*)** compares the inhibitory effect of DMF on CD62E with that on CD54 expression. At 25 and 40 μM, DMF inhibited CD62E but not CD54 expression. A minimal reduction of CD54 expression was seen at 140 μM DMF. In order to see a significant inhibition of CD54 expression by DMF, concentrations had to be raised to 700 μM (data not shown).

A 60 min preincubation period with DMF is sufficient to prevent subsequent TNF-induced CD62E expression In the next set of experiments, we tested onset and duration of DMF effects. HUVEC were pretreated with DMF for 1, 10, or 60 min. DMF was then removed, and cells were rested for various periods of time up to 24 h and then stimulated with TNF. Following a 1 or 10 min DMF preincubation, subsequent TNF-induced CD62E expression was not inhibited. In contrast, after a 60 min

preincubation with DMF subsequent TNF-induced CD62E expression was inhibited for up to 24 h and returned to normal thereafter. Results of a 4 h resting period are shown in **Fig 3**.

DMF inhibits TNF-induced CD62E mRNA expression CD62E and CD54 mRNA expression was analyzed by northern blot analysis (**Fig 4**). HUVEC were preincubated with DMF or FA and then stimulated with TNF for various times. Due to the different inducibility of the two proteins tested, different time courses were chosen. CD62E mRNA is inducible by TNF within the first 3 h. Therefore, mRNA from HUVEC stimulated with TNF for 1 and 3 h and pretreated with DMF or FA was compared. After 1 h of stimulation with TNF, CD62E mRNA was detectable in HUVEC preincubated with FA. After 3 h of stimulation, the detectable CD62E mRNA signal increased nearly 3-fold. After preincubation with DMF, after 1 or 3 h of TNF stimulation, CD62E mRNA was nearly undetectable, resulting in a dramatically reduced CD62E protein expression detected by FACS analysis. On the other hand, CD54 mRNA is inducible over a more prolonged period of time, reflecting the transcriptional control exerted by different transcription factors. Therefore, HUVEC were stimulated for up to 18 h with TNF. After preincubation with FA, CD54 mRNA was strongly detectable after 3 and 12 h of stimulation; after 18 h the signal intensity slowly decreased. HUVEC preincubated with DMF showed a reduced amount of mRNA after 3 h of incubation, but after 12 or 18 h the detectable amount of mRNA compared with the FA preincubated cells was equal or even slightly increased.

DMF inhibits NF-κB but not AP-1-dependent transcription HUVEC transfected with the CD62E promoter or constructs containing NF-κB or AP-1 binding sites were treated with TNF with or without 40 μM DMF (**Fig 5**). Cells transfected with the CD62E promoter showed a mean 8-fold increase in luciferase activity following a 2 h TNF stimulation, which was inhibited by DMF ($p < 0.05$). The level of inhibition by 40 μM DMF seen in the CD62E promoter construct roughly corresponded to the DMF-induced inhibition of CD62E protein expression at the same concentration. Similarly, using an NF-κB-responsive construct, the TNF-induced increase in luciferase activity was significantly inhibited by DMF. In contrast, using an AP-1-responsive construct, DMF had no effect on TNF-induced luciferase activity.

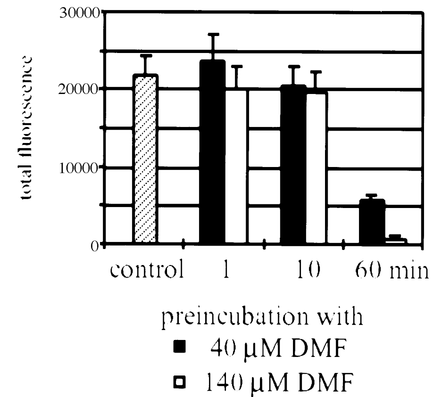


Figure 3. A 60 min preincubation with DMF inhibits subsequent TNF-induced CD62E expression. Summary of five independent experiments. Total fluorescence values are calculated as total fluorescence values ± SD as described in *Materials and Methods*. After preincubation with DMF for the indicated times, cells were washed and cultured in medium alone for another 4 h. Cells were then stimulated with TNF for 4 h. *Hatched bar*, CD62E expression of TNF-stimulated cells; *closed bars*, pretreatment of 40 μM DMF plus TNF; *open bars*, pretreated cells with 140 μM DMF plus TNF.

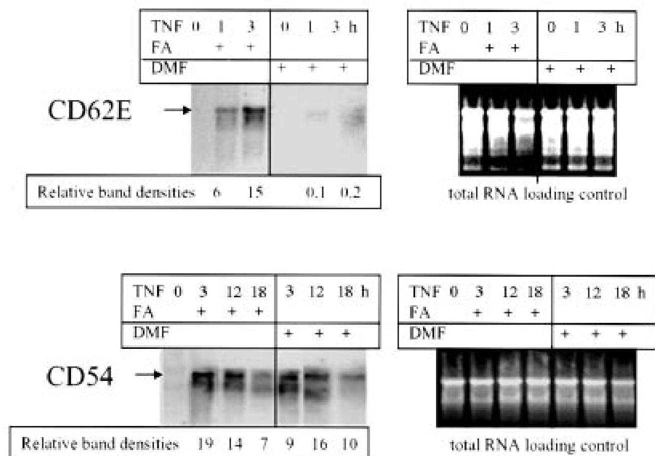


Figure 4. DMF inhibits CD62E mRNA expression. RNA was isolated from 10^6 HUVEC after preincubation with 40 μM DMF or FA and stimulation with TNF for the indicated times. After incubation, total RNA was prepared and northern blotting with probes against CD62E and CD54 was performed. Control of equal RNA content was performed by agarose gel electrophoresis of aliquots of the prepared RNA samples, visualized with ethidium bromide. Relative band densities calculated by the Kodak electrophoresis documentation and analysis system 120 are shown.

DISCUSSION

Recruitment of white blood cells from the circulation into tissues strongly depends on the expression of endothelial adhesion molecules. Many of these molecules are inducible by TNF. Indeed, several inflammatory diseases benefit from an inhibition of TNF-mediated cell activation (Neurath *et al*, 1996; Paleolog *et al*, 1996). Here, we demonstrate that DMF inhibits TNF-induced endothelial cell activation, as evidenced by reduced endothelial CD62E expression in psoriatic skin *in vivo* and reduced CD62E mRNA and protein expression *in vitro*. Likewise, DMF inhibited transcription of a CD62E promoter construct and of an NF-κB reporter construct to a similar extent, but not of an AP-1 construct. This is in line with the fact that CD62E promoter activation mainly depends on NF-κB (Schindler and Baichwal, 1994; Read *et al*, 1997) and allows the conclusion that DMF inhibited CD62E expression at the transcriptional level in an NF-κB-dependent way.

The specificity of DMF to inhibit CD62E expression is dose dependent and seen at concentrations ≦70 μM. At these concentrations DMF did not block CD54 expression. In contrast to CD62E expression, which is mainly under the control of NF-κB, regulation of CD54 expression is more complex (Roebuck and Finnegan, 1999). The CD54 promoter is activated by three major pathways, through NF-κB, AP-1, and through JAK/STAT pathways, which partly interact and partly overlap. TNF activates CD54 gene expression through NF-κB and AP-1, whereas JAK/STAT activation occurs through interferons. In cell culture, CD54 mRNA expression was slightly reduced 3 h following TNF stimulation reflecting the NF-κB-dependent early phase of CD54 induction. At later time points, no inhibition of CD54 mRNA could be detected. In contrast, after 12 and 18 h the amount of detectable CD54 mRNA was slightly higher in the DMF-pretreated cells as determined by relative band densities. It is known that the AP-1-dependent transcription of CD54 starts at a later time point; therefore the mRNA levels at 12 and 18 h of incubation reflect the not inhibited AP-1-dependent gene transcription. CD62E mRNA expression was nearly totally inhibited during the whole time course. Corresponding to the effects of DMF on the mRNA expression of CD54, CD54 protein expression was not inhibited by ≦70 μM DMF. It is thus likely

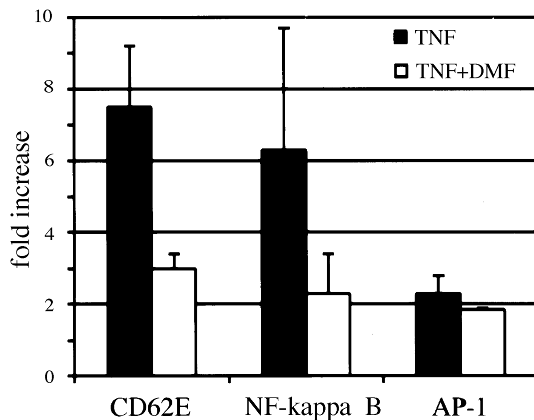


Figure 5.DMF inhibits expression of a CD62E NF-κB reporter construct. HUVEC were transfected with a 1.3 kb construct of the CD62E promotor, a construct containing three consensus NF-κB repeats, or a construct containing three consensus AP-1 repeats using a standard Ca^{2+} phosphate precipitation technique as described in *Materials and Methods*. Cells were cotransfected with a pSV40-β-galactosidase vector to control for transfection efficiency. Two days after transfection, cells were stimulated with 10 ng per ml TNF (*closed bars*) or treated with TNF and 40 μM DMF (*open bars*) for 2 h. Luciferase activities were normalized according to their β-galactosidase activity. The variation of β-galactosidase activity within individual experiments was < 10%. Values shown are the mean increase of relative luciferase activities ± SD compared to unstimulated cells, summary of 10 independent experiments. Statistical analysis was performed using Student's *t* test. CD62E, $p < 0.05$; NF-κB, $p < 0.05$; AP-1, not significant.

that the reduced NF-κB-dependent transcription is balanced by AP-1. Indeed, transcription of an AP-1 reporter construct was not affected by DMF. Moreover, this finding correlated with our observation *in vivo*, where CD54 expression remained unchanged throughout a treatment period of 5 wk.

DMF readily hydrolyzes into MHF and methanol. Therefore, MHF and not DMF was believed to mediate the biologic response (Nibbering *et al*, 1993). Our experiments using endothelium, however, demonstrated that MHF, at equimolar concentrations to DMF, did not inhibit CD62E expression. Thus, in the context of endothelial cells, MHF is unlikely to be the active metabolite of DMF. We wish to point out that effects of FAE on endothelial cells differ from those on lymphocytes and keratinocytes, where both DMF and MHF appear equally effective (Thio *et al*, 1994; de Jong *et al*, 1996).

The mechanism by which DMF transmits signals to endothelial cells is not yet understood. Among several possibilities, DMF may inhibit the TNF–TNF receptor interaction. This is unlikely, however, because the TNF-induced CD54 expression is not blocked by DMF. Our finding that a 60 min preincubation with DMF followed by washout is sufficient to maintain endothelial cells unresponsive to TNF raises another possibility. DMF may be taken up into cells and lead to a persisting inhibition of signal transduction. Interestingly, DMF is known to interfere with the cellular glutathione metabolism (Miller *et al*, 1993; Nelson *et al*, 1999). As glutathione is known to interfere with NF-κB translocation, this could be a possible mechanism for the observed CD62E inhibition by DMF. As another possibility, DMF might inhibit nuclear translocation of NF-κB p50/p65 heterodimers as CD62E expression depends on these heterodimers. Indirect evidence for this assumption comes from recent work by Vandermeeren *et al* who found that DMF inhibited nuclear entry of NF-κB p50 in dermal fibroblasts (Vandermeeren *et al*, 2001). Importantly, Vandermeeren *et al* found translocation of NF-κB p65 not to be affected. Therefore, results obtained in fibroblasts cannot easily be transposed to effects of DMF seen in endothelial cells. In endothelium, as in many other cell types, p50/p65 is shuttled as a complex; thus translocation of both proteins should be affected by DMF. It is unlikely that DMF inhibits CD62E expression by inhibiting translocation of p50 homodimers, because p50 homodimers have the reverse effect – they inhibit p50/p65 DNA binding and transcription (Baer *et al*, 1998).

CaMF, EHF, and MHF exhibit an inhibitory effect on endothelial functions only at 700 μM. At this concentration, even interferon-γ-induced HLA-DR expression on endothelial cells is inhibited (data not shown). As HLA-DR expression is independent from NF-κB signaling, it may be assumed that this high concentration of FAE inhibits endothelial cell activation by pathways other than NF-κB. With regard to DMF, specificity for NF-κB-dependent genes is lost at concentrations of approximately 100 μM. We thus conclude that with regard to the inhibition of endothelial activation DMF is the therapeutic active FAE. In endothelium, DMF appears to have a dose-dependent specificity for NF-κB. It thus can be assumed that the therapeutic potency of DMF in psoriasis is due to the combined effect of DMF on functions of endothelial cell, keratinocyte, lymphocyte, and dendritic cell (Thio *et al*, 1994; Vandermeeren *et al*, 1997; Ockenfels *et al*, 1998; Zhu and Mrowietz, 2001).

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