



## Effect of Colchicine and Cytokines on *MEFV* Expression and C5a Inhibitor Activity in Human Primary Fibroblast Cultures

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**Key words:** familial Mediterranean fever, *MEFV*, C5a inhibitor

### Abstract

**Background:** Familial Mediterranean fever is an autosomal recessive disease characterized by sporadic attacks of inflammation affecting the serosal spaces. The gene associated with FMF (*MEFV*), mainly expressed in neutrophils, was recently found to be expressed also in primary cultures of serosal origin (peritoneal and synovial fibroblasts). A C5a inhibitor, previously detected in normal serosal fluids, was recently identified in serosal cultures as well, and was found to be deficient in serosal fluids and cultures obtained from FMF patients.

**Objective:** To investigate the effect of colchicine (the main therapeutic agent for FMF patients) and certain inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\alpha$ , IFN- $\gamma$ ) on *MEFV* expression and C5a inhibitor activity in neutrophils and primary peritoneal fibroblast cultures.

**Methods:** Human primary peritoneal fibroblast cultures and neutrophils were studied for *MEFV* expression and C5a inhibitor activity, using reverse transcription-polymerase chain reaction and C5a-induced myeloperoxidase assay, respectively, in the presence and absence of colchicine and cytokines.

**Results:** *MEFV* expression in neutrophils was high and could not be induced further. Its expression in the peritoneal fibroblasts was lower than in neutrophils and could be induced using colchicine and cytokines parallel with induction of C5a inhibitor activity. Semi-quantitative RT-PCR assays enabled estimation of *MEFV* induction by the cytokines at 10–100-fold and could not be further increased by concomitant addition of colchicine.

**Conclusion:** Serosal tissues, which are afflicted in FMF, express colchicine and cytokine-inducible *MEFV* and contain inducible C5a inhibitor activity. The relation between the ability of colchicine to induce *MEFV* and C5a inhibitor activity, and its efficacy in FMF treatment, require further investigation.

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FMF is an autosomal recessive disease characterized by sporadic attacks of inflammation affecting the serosal spaces [1], and occurs most frequently in four populations of eastern Mediterranean descent: non-Ashkenazi Jews, Armenians, Turks, and Arabs. Clinical features include recurrent bouts of fever and abdominal, pleural or joint pain, with evidence of systemic and localized inflammation that can be efficiently prevented by the anti-inflammatory agent colchicine.

We have previously suggested that a C5a/interleukin-8 inhibitor plays a role in the etiology of FMF [2–4]. This serine protease, which neutralizes the complement fragment C5a and the cytokine IL-8 – both major inflammatory mediators – should prevent inappropriate episodes of inflammation that would otherwise result from release of these powerful mediators [5–9]. It is produced by synovial and peritoneal fibroblasts but not by skin fibroblasts [10], and its activity is reduced in serosal fluids and serosal cultures of FMF patients [7–9,11], suggesting that it plays a role in the regulation of the inflammatory attacks characteristic of this disease.

The gene associated with FMF (designated *MEFV*) was recently cloned [12,13]. It is expressed mainly in neutrophils and encodes a novel protein, called pyrin or marenostrin. Computational studies of its protein sequence revealed homology to a number of nuclear factors [13]. However, further studies failed to localize it to the nucleus, and transcription activation activity or self-interaction activity could not be detected [14,15]. Based on these studies, pyrin was proposed to play a role in the regulation of the inflammatory process in a mechanism as yet unknown. In addition to its expression in neutrophils, *MEFV* mRNA could be detected in induced myelomonocytic cell lines and several other malignant cell lines [11,14,16]. We also showed that *MEFV* is expressed in peritoneal and skin human primary fibroblast cultures, representing the main tissues afflicted in FMF [11]. Its expression was lower than in neutrophils and could be further induced by

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FMF = familial Mediterranean fever

RT-PCR = reverse transcription-polymerase chain reaction

IL = interleukin

phorbol myristate acetate and IL-1 $\beta$ . C5a inhibitor activity was also detected in these cultures and could be further induced [11].

Colchicine, the only effective agent in FMF therapy, is highly concentrated in neutrophils. Since these cells are the main ones expressing the FMF gene, we wished to investigate whether colchicine can exert a direct agonistic effect on their gene as well [17]. In the present paper we describe the effect of colchicine and several inflammatory cytokines on *MEFV* expression and on C5a inhibitor activity in normal human peritoneal fibroblast cultures and in normal human neutrophils.

## Materials and Methods

Recombinant C5a was purchased from Sigma (St. Louis, MO, USA) and was dissolved in d-H<sub>2</sub>O containing 2.5 mg/ml of bovine serum albumin. Media and buffers (Dulbecco's phosphate-buffered saline, Hank's balanced salt solution, F-10 medium and supplements were obtained from Biological Industries, Beit HaEmek, Israel), and fetal calf serum from Gibco (Grand Island, NY, USA). IL-1 $\beta$ , IL-6, interferon- $\gamma$ , IFN- $\alpha$  and tumor necrosis factor- $\alpha$  were purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). Colchicine (Sigma) was dissolved in d-H<sub>2</sub>O. All other chemicals (Sigma) were of reagent grade.

### Cell cultures

Peritoneal biopsies were obtained from patients undergoing surgical procedures because of hernia or suspected appendicitis. Primary cultures were established and maintained by a modification of the method of Matzner et al. [9] as described [11]. Freshly passaged fibroblasts were prepared for an experiment by growing to confluence in T-25 flasks. In all experiments, the number of cells was  $0.5 \times 10^6$  cells/T-25 flask. After 4 days, the supernatant and non-adherent cells were removed, the monolayers were washed, and fresh medium (F-10) supplemented with fetal calf serum was added. Fibroblasts were incubated without (control) or with IL-1 $\beta$  (10 ng/ml), TNF- $\alpha$  (10 ng/ml), IFN- $\alpha$  (1 ng/ml), IFN- $\gamma$  (10 ng/ml) or colchicine (0.02, 0.2, 2, 10, 50  $\mu$ M). Fibroblasts were also incubated with a combination of either one of the inflammatory cytokines (TNF- $\alpha$  10 ng/ml, IFN- $\alpha$  1 ng/ml, or IL-1 $\beta$  10 ng/ml) with 10 or 50  $\mu$ M colchicine to investigate the possibility of synergistic or antagonistic effect on *MEFV* expression. Experiments concomitantly assaying *MEFV* expression and C5a inhibitor activity were carried out in two sets: One set was harvested after 24 hours of incubation for RNA extraction and RT-PCR analysis. For the second set, the medium was replaced with serum-free medium for an additional 24 hours; supernatants were then harvested for analysis of C5a-induced myeloperoxidase release.

IFN = interferon

TNF = tumor necrosis factor

### RT-PCR

Total RNA from the various cultured fibroblasts was prepared using a highly pure RNA isolation kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. All samples included the same amount of RNA (4  $\mu$ g RNA/sample) in the RT reaction. The cDNA was synthesized using hexamer primer and the superscript II RNase H reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). The cDNA was amplified with Supertherm DNA polymerase (SRP-801, Roche Diagnostics GMBH, Mannheim, Germany) and primers designed from exon 8 and 10 or 9 and 10 of *MEFV*. The primer sequences from exons 8 and 10 were: Forward 5'-TTCAATGTTC-CAGAGCTG-3'; Reverse 5'-TGTAGTCCACGAAGATGC-3', respectively. The primers from exons 9 and 10 were: Forward 5'-GATTGGCGCTCAGGCACATGCTGTTA-3'; Reverse 5'-GTCTGGGGAAACGCTGGACGCCTGGTA-3', respectively. Mixtures were incubated in a thermocycler (MJ Research Inc., Watertown, MA, USA) under the following conditions: 1 cycle of 2 minutes at 95°C followed by 35 cycles each consisting of 30 seconds at 95°C, 30 sec at 55°C, and 30 sec at 72°C, followed at the end by 1 cycle of 10 minutes at 72°C. The amplified products were separated by electrophoresis on a 2% agarose gel. Ethidium bromide staining of the agarose gel was used to detect the amplified fragments (420 basepairs and 351 bp, respectively). Amplification of a fragment of the housekeeping gene  $\beta$  actin (220 bp fragment) was used as positive control for successful amplification of the cDNA. Negative control included replacement of the cDNA mixture with H<sub>2</sub>O in the PCR reaction.

### Myeloperoxidase release

C5a-induced MPO release from neutrophils was used to measure the presence of C5a inhibitor activity in conditioned media of human primary fibroblast cultures, as described [10]. Briefly, 50  $\mu$ l of 5 nmol/L rC5a and 50  $\mu$ l of the conditioned media derived from 3 wells of a 24-well dish were each loaded into 3 wells in a 96-well microtiter plate and incubated for 20 min at 37°C. Twenty-five microliters of freshly prepared human neutrophils ( $4 \times 10^6$  cells/ml in HBSS/25 mM Hepes/0.25% bovine serum albumin) that had been incubated with 5  $\mu$ g/ml cytochalasin B for 10 min at 37°C were added to each well. Degranulation was allowed to proceed for 10 min at 37°C and MPO release was then measured using an enzyme-linked immunosorbent assay reader (Dynatech MR 5000). The results were corrected for MPO release in the absence of rC5a and compared with those obtained in wells containing rC5a in the absence of a putative source of C5a inhibitor.

### Calculations

MPO release was corrected for enzyme release in the absence of rC5a. Inhibition of MPO release was calculated in comparison

bp = basepairs

MPO = myeloperoxidase

rC5a = recombinants C5a

with its release in wells containing rC5a in the absence of a putative source of C5a inhibitor ("culture") as follows:

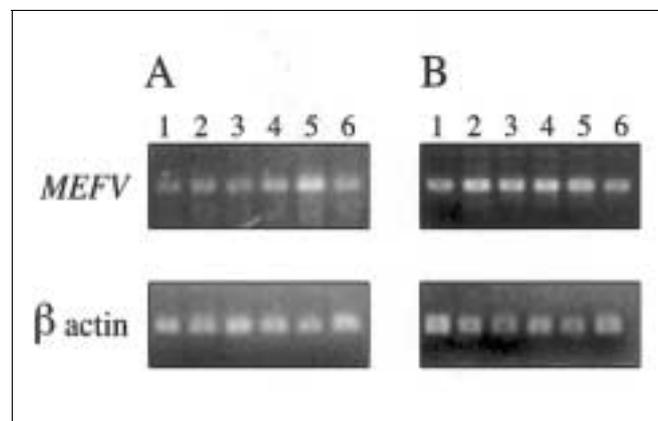
$$\text{Percent inhibition} = 100 \times 1 - \frac{\text{MPO release with culture}}{\text{MPO release without culture}}$$

Induced culture medium was always assayed in comparison with culture medium in the absence of an inducer. Results are expressed as mean  $\pm$  SEM. Significance was determined by the paired *t*-test.

## Results

The effect of colchicine on *MEFV* expression in peritoneal fibroblasts is shown in Figure 1. Colchicine could induce *MEFV* expression in peritoneal fibroblasts in a dose-dependent manner [Figure 1A]. At 0.02  $\mu\text{M}$  (the therapeutic plasma concentration in colchicine-treated FMF patients) the induction was minimal, reaching maximal effect at 10  $\mu\text{M}$ . At higher colchicine concentration, inhibition of *MEFV* expression was observed [Figure 1A, lane 6].

In neutrophils, where *MEFV* expression is generally higher than in peritoneal fibroblasts, significant induction with colchicine could not be observed [Figure 1B]. It is of interest

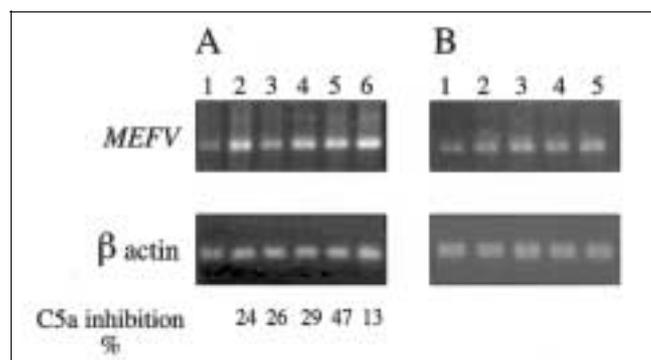


**Figure 1.** Effect of colchicine on *MEFV* expression in primary peritoneal fibroblast cultures **[A]** and neutrophils **[B]**. Peritoneal fibroblasts were harvested after 24 hours and neutrophils after 3 hours of incubation, respectively. RT-PCR analysis was performed on total RNA using two pairs of primers. Primers from exon 9 and 10 were used to amplify peritoneal fibroblast cDNA (351 bp) and primers from exon 8 and 10 were used to amplify neutrophil cDNA (420 bp) as described in the Materials and Methods section. Six different cultures of peritoneal fibroblasts were studied. **[A]** Primary human peritoneal fibroblasts. Lane 1: normal peritoneal fibroblasts. Lanes 2–6: colchicine-induced peritoneal fibroblasts. (Colchicine dose: lane 2: 0.02  $\mu\text{M}$ , lane 3: 0.2  $\mu\text{M}$ , lane 4: 2  $\mu\text{M}$ , lane 5: 10  $\mu\text{M}$ , lane 6: 50  $\mu\text{M}$ ). **[B]** Human neutrophils. Lane 1: normal human neutrophil. Lanes 2–6: colchicine-induced neutrophils. (Colchicine dose: lane 2: 0.02  $\mu\text{M}$ , lane 3: 0.2  $\mu\text{M}$ , lane 4: 2  $\mu\text{M}$ , lane 5: 10  $\mu\text{M}$ , lane 6: 50  $\mu\text{M}$ ).

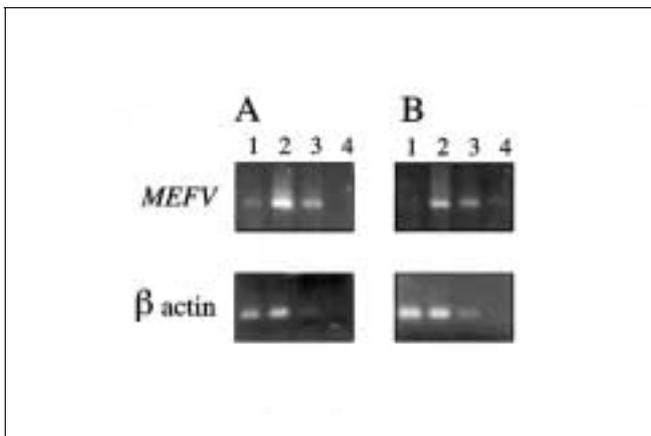
to note that even the minimal induction, when observed, was inhibited at a high colchicine concentration of 50  $\mu\text{M}$  [Figure 1B, lane 6], suggesting a toxic effect.

The effect of various agents on the induction of *MEFV* expression and C5a inhibitor activity is shown in Figure 2. Colchicine and the cytokines studied (IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\alpha$ , IFN- $\gamma$ ) induced both *MEFV* expression and C5a inhibitor activity in the serosal fibroblast cultures [Figure 2A]. TNF- $\alpha$  was the strongest inducer of C5a inhibitor activity [Figure 2A, lane 5, bottom]. *MEFV* expression was induced efficiently by colchicine, as well as by TNF- $\alpha$ , IFN- $\gamma$  and IL-1 $\beta$ . IFN- $\alpha$  was the least efficient inducer [Figure 2A, lane 3, top]. No enhancing effect of colchicine and the various cytokines on *MEFV* expression in neutrophils could be documented [Figure 2B] (C5a inhibitor cannot be demonstrated in neutrophils by the method used).

Semi-quantitative RT-PCR analysis of TNF- $\alpha$  and IFN- $\gamma$  treated peritoneal cultures revealed a 10 to 100-fold increase



**Figure 2.** Effect of colchicine and inflammatory cytokines on *MEFV* expression and C5a inhibitor activity. RT-PCR analysis was performed using two pairs of primers. Primers from exon 9 and 10 were used to amplify peritoneal fibroblast cDNA (351 bp) and primers from exon 8 and 10 were used to amplify neutrophil cDNA (420 bp) as described in Materials and Methods. Three different cultures of peritoneal fibroblasts were studied. C5a inhibitor activity is shown at the bottom. **[A]** Primary human peritoneal fibroblasts. Lane 1: normal peritoneal fibroblasts. Lanes 2–6: induced peritoneal fibroblasts (inducer: lane 2: 10  $\mu\text{M}$  colchicine, lane 3: 1 ng/ml IFN- $\alpha$ , lane 4: 10 ng/ml IL-1 $\beta$ , lane 5: 10 ng/ml TNF- $\alpha$ , lane 6: 10 ng/ml IFN- $\gamma$ ). **[B]** Human neutrophils. Lane 1: normal human neutrophils. Lanes 2–5: induced neutrophils, (inducer: lane 2: 10  $\mu\text{M}$  colchicine, lane 3: 10 ng/ml IL-1 $\beta$ , lane 4: 10 ng/ml TNF- $\alpha$ , lane 5: 10 ng/ml IFN- $\gamma$ ). C5a inhibitor activity was measured as percent of inhibition of rC5a-induced release of MPO from neutrophils as described, and expressed as the mean of at least 3 experiments.  $A_{490}$  readings for rC5a-induced MPO release of cultures without an inducer was  $0.30 \pm 0.05$ . C5a inhibition observed in these non-induced normal peritoneal fibroblast culture media was  $6 \pm 4\%$  (6 experiments from 3 different cultures, passages 5,6,8).  $A_{490}$  readings for control rC5a-induced MPO release without conditioned medium were  $0.32 \pm 0.06$ .  $P < 0.05$  for all the induced, compared with the non-induced, cultures.



**Figure 3.** Semi-quantitative RT-PCR assay of *MEFV* expression. RT-PCR analysis was performed using primers from exons 9 and 10 as described in Materials and Methods. The size of the amplified fragment was 351 bp. **[A]** Induction with  $\text{TNF-}\alpha$ . Lane 1: normal peritoneal fibroblasts. Lane 2: normal peritoneal fibroblasts induced for 24 hours with 10 ng/ml  $\text{TNF-}\alpha$ ; undiluted sample. Lane 3:  $\text{TNF-}\alpha$  induced peritoneal fibroblasts; cDNA diluted 1:10. Lane 4:  $\text{TNF-}\alpha$  induced peritoneal fibroblasts; cDNA diluted 1:100. **[B]** Induction with  $\text{IFN-}\gamma$ . Lane 1: normal peritoneal fibroblasts. Lane 2: normal peritoneal fibroblasts induced for 24 hr with 10 ng/ml  $\text{IFN-}\gamma$ ; undiluted sample. Lane 3:  $\text{IFN-}\gamma$  induced peritoneal fibroblasts; cDNA diluted 1:10. Lane 4:  $\text{IFN-}\gamma$  induced peritoneal fibroblasts; cDNA diluted 1:100.

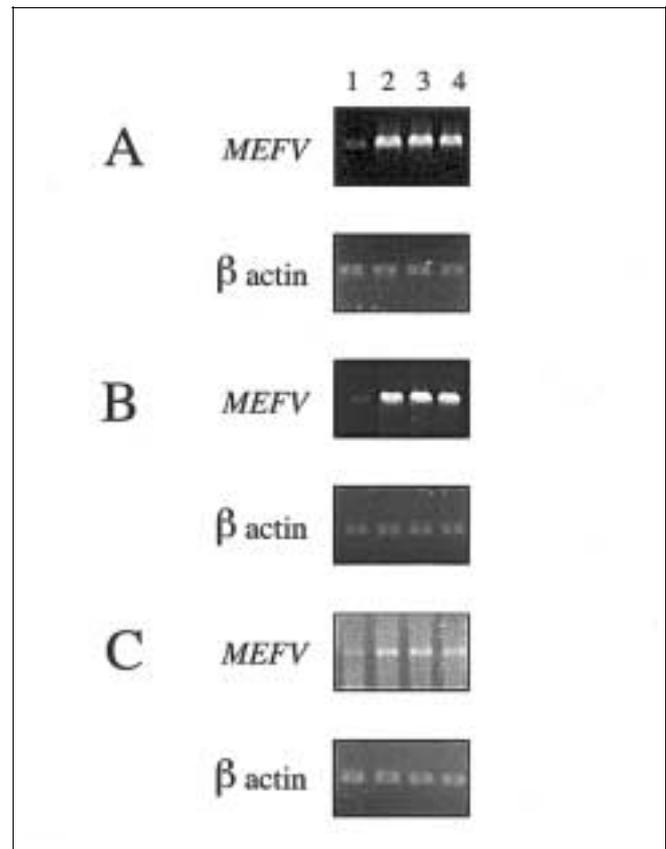
All undiluted samples included the same amount of RNA in the RT reaction.

in *MEFV* expression as compared with non-induced cultures [Figure 3]. This suggests that serosal tissue *MEFV* can respond to an inflammatory stimulus.

We further studied the possible additive effect of colchicine on *MEFV* expression in serosal cultures induced by various cytokines [Figure 4]. In Figure 4A,  $\text{IL-1}\beta$  was the inducer without (lane 2) or with 10  $\mu\text{M}$  or 50  $\mu\text{M}$  colchicine (lanes 3 and 4, respectively). The same experiment was performed with  $\text{TNF-}\alpha$  [Figure 4B], and  $\text{IFN-}\alpha$  [Figure 4C]. In all three experiments, the induction by the cytokine (lanes 2) could not be further induced, nor inhibited, by addition of colchicine.

## Discussion

*MEFV* was recently identified as a disease-related gene in patients with FMF. It is a member of a family of highly conserved genes that includes nuclear effector molecules and nucleic acid-binding proteins that regulate inflammation, hematopoiesis, oncogenesis and embryonic development [18–20]. However, recent studies failed to localize it to the nucleus or to detect transcription activity [14], and its function and regulatory roles are still obscure. Expression of *MEFV* mRNA was originally described in peripheral blood neutrophils [12,13]. Later it was also detected in eosinophils and monocytes, and at low level expression in  $\text{CD19+}$ B and  $\text{CD3+}$ T lymphocytes [14]. The



**Figure 4.** Induction of *MEFV* expression in human peritoneal fibroblasts by a mixture of colchicine and an inflammatory cytokine. RT-PCR analysis was performed as described in Figure 1. **[A]** Induction with colchicine and  $\text{IL-1}\beta$ . Lane 1: normal peritoneal fibroblasts. Lane 2:  $\text{IL-1}\beta$  (10 ng/ml) induced peritoneal fibroblasts. Lane 3:  $\text{IL-1}\beta$  (10 ng/ml) and 10  $\mu\text{M}$  colchicine induced peritoneal fibroblasts. Lane 4:  $\text{IL-1}\beta$  (10 ng/ml) and 50  $\mu\text{M}$  colchicine induced peritoneal fibroblasts. **[B]** Induction with colchicine and  $\text{TNF-}\alpha$ . Lane 1: normal peritoneal fibroblasts. Lane 2:  $\text{TNF-}\alpha$  (10 ng/ml) induced peritoneal fibroblasts. Lane 3:  $\text{TNF-}\alpha$  (10 ng/ml) and 10  $\mu\text{M}$  colchicine induced peritoneal fibroblasts. Lane 4:  $\text{TNF-}\alpha$  (10 ng/ml) and 50  $\mu\text{M}$  colchicine induced peritoneal fibroblasts. **[C]** Induction with colchicine and  $\text{IFN-}\alpha$ . Lane 1: normal peritoneal fibroblasts. Lane 2:  $\text{IFN-}\alpha$  (1 ng/ml) induced peritoneal fibroblasts. Lane 3:  $\text{IFN-}\alpha$  (1 ng/ml) and 10  $\mu\text{M}$  colchicine induced peritoneal fibroblasts. Lane 4:  $\text{IFN-}\alpha$  (1 ng/ml) and 50  $\mu\text{M}$  colchicine induced peritoneal fibroblasts.

pre-promyelocytic cell line HL60 upon granulocytic and monocytic differentiation and bone marrow leukocytes at the myelocyte stage expressed high levels of *MEFV* [11,14–16]. Among normal tissues it was detected in spleen, lung and muscle [14]. Studies of tumor cell lines revealed relatively high *MEFV* mRNA expression in myeloid leukemia, colon cancer and prostate cancer cell lines [14]. The clinical relevance of *MEFV* expression in the malignant cell lines is not known. Recently we showed

that *MEFV* mRNA is expressed in synovial, peritoneal – and to a lesser extent skin – primary cultured human fibroblasts [11].

*MEFV* mRNA could be induced upon *in vitro* stimulation of monocytes with the pro-inflammatory cytokines IFN- $\gamma$ , TNF- $\beta$ , lipopolysaccharides and IFN- $\gamma$  but not by anti-inflammatory stimuli [16]. In a single neutrophil preparation with low *MEFV* expression, the gene could be upregulated by IFN- $\gamma$  and the combination of IFN- $\alpha$  and colchicine but not by colchicine alone, nor by IFN- $\alpha$ , C5a, TNF- $\alpha$ , granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor or IL-4 [16]. This single experiment is exceptional since *MEFV* expression in neutrophils is usually high and cannot be further induced as demonstrated in the present paper. On the other hand, we have recently shown that phorbol myristate acetate and IL-1 $\beta$  could further induce *MEFV* expression in skin and peritoneal fibroblasts [11], which are used by us as representatives of the tissues afflicted in FMF. In the present work we expanded those studies to investigate the effect of colchicine and various cytokines on *MEFV* expression and C5a inhibitor activity in primary peritoneal fibroblast cultures.

Our results show that *MEFV* expression in the serosal cultures is lower than in neutrophils but can be further induced by colchicine and various pro-inflammatory cytokines. *MEFV* induction was followed by induction of C5a inhibitor activity. Colchicine induced *MEFV* expression in the serosal cultures in a dose-dependent manner, starting at 0.02  $\mu$ M (concentration found in the plasma of colchicine-treated patients) and reaching maximal effect at 10  $\mu$ M. Neither colchicine nor the cytokines tested showed *MEFV* gene induction in neutrophils. Our results are in line with those of Centola et al. [16] who demonstrated such induction only in a single neutrophil preparation with basic low *MEFV* expression.

Interestingly, colchicine and IFN- $\alpha$ , the only therapeutic agents known to ameliorate FMF inflammatory attacks [21–23], as well as TNF- $\alpha$ , IFN- $\gamma$  and IL-1 $\beta$ , which upregulate *MEFV* expression in primary serosal fibroblast cultures, also upregulate C5a inhibitor activity in these culture media. Thus, appreciating the anti-inflammatory potential of the C5a/IL-8 inhibitor, one might define *MEFV* as an inhibitor of inflammation. The fact that *MEFV* message levels in serosal cells are increased by colchicine and pro-inflammatory cytokines may suggest that the gene functions, among other activities, by upregulating the C5a/IL-8 inhibitor activity, thus aborting undesired inflammatory reactions in serosal spaces. If this model of therapeutic effect is proved by others, then upregulating *MEFV* mRNA by other agents – especially IFN- $\gamma$ , but potentially also TNF- $\alpha$  and IL-1 $\beta$ , as shown by us and by Centola et al. [16] – may be worth evaluating as an alternative treatment for patients suffering from colchicine-resistant FMF attacks.

In summary, the data presented here support the hypothesis that *MEFV* is an inflammatory regulator, not only in polymorphonuclear leukocytes but also in cells of serosal origin, where FMF attacks occur. *MEFV* is expressed in serosal fibroblasts, in accordance with induction of C5a inhibition,

and both the gene expression and the anti-inflammatory activity are upregulated by colchicine and cytokines. *MEFV* mediates probably more than one inflammatory pathway. Centola and colleagues [16] suggested a Th1-responsive negative-feedback loop in myeloid cells. We suggest a C5a inhibitor-responsive positive-feedback loop in the serosal tissues themselves, and others suggest regulation of lipocortins [24] and TNF [25] in familial Mediterranean fever. Thus, a broader role of *MEFV* as an inflammatory regulator in FMF and other diseases with an inflammatory component might be suggested.

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