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

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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-1β, TNF-α, IFN-α, IFN-γ) 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 affected 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 affected in FMF [11]. Its expression was lower than in neutrophils and could be further induced by

* Decreased. Prof. Matzner died tragically in a plane crash on 24 November 2001, returning home from a scientific meeting in Germany.

RT-PCR = reverse transcription-polymerase chain reaction
IL = interleukin
phorbol myristate acetate and IL-1β. 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$_2$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-1β, IL-6, interferon-γ, IFN-α and tumor necrosis factor-β were purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). Colchicine (Sigma) was dissolved in d-H$_2$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 0.5x10^6 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-1β (10 ng/ml), TNF-α (10 ng/ml), IFN-α (1 ng/ml), IFN-γ (10 ng/ml) or colchicine (0.2, 0.2, 2, 10, 50 µM). Fibroblasts were also incubated with a combination of either one of the inflammatory cytokines (TNF-α 10 ng/ml, IFN-α 1 ng/ml, or IL-1β 10 ng/ml) with 10 or 50 µM 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.

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 µg 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'-TTCATGTTC-CAGAGCTG-3'; Reverse 5'-TGTAGTCCAGAAGAGC-3', respectively. The primers from exons 9 and 10 were: Forward 5'-GATTTGCGCCTCAGGCACTGCTTGTA-3'; Reverse 5'-GTCGGGGGAAACGCAGCGGCTGGTA-3', respectively. Mixtures were incubated in a thermocycler (MI 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 by 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 β 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$_2$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 µl of 5 nmol/L rC5a and 50 µl 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 (4x10^6 cells/ml in HBSS/25 mM Hepes/0.25% bovine serum albumin) that had been incubated with 5 µg/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

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IFN = interferon
TNF = tumor necrosis factor
bp = basepairs
MPO = myeloperoxidase
rC5a = recombinant C5a
with its release in wells containing rC5a in the absence of a putative source of C5a inhibitor (“culture”) as follows:

\[
\text{Percent inhibition} = 100 \times \left(1 - \frac{\text{MPO release with culture}}{\text{MPO release without culture}}\right)
\]

Induced culture medium was always assayed in comparison with culture medium in the absence of an inducer. Results are expressed as mean ± 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 μM (the therapeutic plasma concentration in colchicine-treated FMF patients) the induction was minimal, reaching maximal effect at 10 μM. 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 to note that even the minimal induction, when observed, was inhibited at a high colchicine concentration of 50 μM [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-1β, TNF-α, IFN-α, IFN-γ) induced both MEFV expression and C5a inhibitor activity in the serosal fibroblast cultures [Figure 2A]. TNF-α 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-α, IFN-γ and IL-1β. IFN-α 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-α and IFN-γ treated peritoneal cultures revealed a 10 to 100-fold increase in C5a expression as compared to control cultures (Figure 2). [A] Primary human peritoneal fibroblasts. Lane 1: normal peritoneal fibroblasts. Lanes 2–6: induced peritoneal fibroblasts (inducer: lane 2: 10 μM colchicine, lane 3: 1 ng/ml IFN-α, lane 4: 10 ng/ml IL-1β, lane 5: 10 ng/ml TNF-α, lane 6: 10 ng/ml IFN-γ). [B] Human neutrophils. Lane 1: normal human neutrophils. Lanes 2–5: induced neutrophils, (inducer: lane 2: 10 μM colchicine, lane 3: 10 ng/ml IL-1β, lane 4: 10 ng/ml TNF-α, lane 5: 10 ng/ml IFN-γ). 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<sub>540</sub> readings for rC5a-induced MPO release of cultures without an inducer was 0.30 ± 0.05. C5a inhibition observed in these non-induced normal peritoneal fibroblast culture media was 6 ± 4% (6 experiments from 3 different cultures, passages 5, 6, 8). A<sub>540</sub> readings for control rC5a-induced MPO release without conditioned medium were 0.32 ± 0.06. P<0.05 for all the induced, compared with the non-induced, cultures.
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, IL-1β was the inducer without (lane 2) or with 10 μM or 50 μM colchicine (lanes 3 and 4, respectively). The same experiment was performed with TNF-α (Figure 4B), and IFN-γ (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 transcriptional 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 CD19+B and 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 IL-1β. Lane 1: normal peritoneal fibroblasts. Lane 2: IL-1β (10 ng/ml) induced peritoneal fibroblasts. Lane 3: IL-1β (10 ng/ml) and 10 μM colchicine induced peritoneal fibroblasts. Lane 4: IL-1β (10 ng/ml) and 50 μM colchicine induced peritoneal fibroblasts. [B] Induction with colchicine and TNF-α. Lane 1: normal peritoneal fibroblasts. Lane 2: TNF-α (10 ng/ml) induced peritoneal fibroblasts. Lane 3: TNF-α (10 ng/ml) and 10 μM colchicine induced peritoneal fibroblasts. Lane 4: TNF-α (10 ng/ml) and 50 μM colchicine induced peritoneal fibroblasts. [C] Induction with colchicine and IFN-γ. Lane 1: normal peritoneal fibroblasts. Lane 2: IFN-γ (1 ng/ml) induced peritoneal fibroblasts. Lane 3: IFN-γ (1 ng/ml) and 10 μM colchicine induced peritoneal fibroblasts. Lane 4: IFN-γ (1 ng/ml) and 50 μM 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-γ, TNF-β, lipopolysaccharides and IFN-γ but not by anti-inflammatory stimuli [16]. In a single neutrophil preparation with low MEFV expression, the gene could be upregulated by IFN-γ and the combination of IFN-α and colchicine but not by colchicine alone, nor by IFN-α, C5a, TNF-α, 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 phosphor myristate acetate and IL-1β 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 μM (concentration found in the plasma of colchicine-treated patients) and reaching maximal effect at 10 μM. 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-α, the only therapeutic agents known to ameliorate FMF inflammatory attacks [21-23], as well as TNF-α, IFN-γ and IL-1β, 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-γ, but potentially also TNF-α and IL-1β, 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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References


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Capsule

Assembling acrosomes

During spermiogenesis (the events from spermatid to spermatozoon), many pro-acrosomal vesicles coalesce into a single acrosomal vesicle that covers much of the cell’s nuclear surface. Defects in acrosome development or function result in fertility problems. Kang-Decker et al. report the identification of a protein involved in acrosome formation. When the Hrb protein was eliminated from mice via homologous recombination, infertile but otherwise normal males developed because the pro-acrosomal vesicles failed to form a single acrosomal vesicle. In addition, spermatozoa decreased in number and displayed reduced motility – likely the result of defects in the midpiece and tail.

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Capsule

Anti-DNA antibodies cross-react with the NR2 glutamate receptor in systemic lupus erythematosus

In systemic lupus erythematosus, antibodies against double-stranded DNA are a major contributor to renal disease. Lorraine et al. have previously demonstrated that the pentapeptide Asp/Glu-Tyr-Glu-Tyr/Ser/Gly is a molecular mimic of double-stranded DNA. This sequence is also present in the extracellular domain of murine and human NMDA (N-methyl-D-aspartate) receptor subunits NR2a and NR2b. The authors now show that the NR2 receptor is recognized by both murine and human anti-DNA antibodies. Moreover, anti-DNA antibodies with this cross-reactivity mediate apoptotic death of neurons in vivo and in vitro. They show that the cerebrospinal fluid of a patient with systemic lupus erythematosus contains these antibodies and also mediates neuronal death via an apoptotic pathway. These observations indicate that lupus antibodies cross-react with DNA and NMDA receptors, gain access to cerebrospinal fluid and may mediate non-thrombotic and non-vasculitic abnormalities of the central nervous system.

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