Expression of ASC in Renal Tissues of Familial Mediterranean Fever Patients with Amyloidosis: Postulating a Role for ASC in AA Type Amyloid Deposition

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Familial Mediterranean fever (FMF) is characterized by recurrent attacks of fever and serositis; in some cases, ensuing amyloidosis results in kidney damage. Treatment with colchicine reduces the frequency and severity of FMF attacks and prevents amyloidosis, although the mechanisms behind these effects are unknown. Pyrin, the protein product of the *MEFV* gene, interacts with ASC, a key molecule in apoptotic and inflammatory processes. ASC forms intracellular speck-like aggregates that presage cell death. Here we show that cell death after ASC speck formation is much slower in nonmyeloid cells than in myeloid cells. Additionally, we demonstrate that colchicine prevents speck formation and show that specks can survive in the extracellular space after cell death. Because we also found that ASC is expressed in renal glomeruli of patients with FMF but not in those of control patients, we posit that high local ASC expression may result in speck formation and that specks from dying cells may persist in the extracellular space where they have the potential (perhaps in association with pyrin) to nucleate amyloid. The fact that speck formation requires an intact microtubule network as shown here could potentially account for the ability of prophylactic colchicine to prevent or reverse amyloidosis in patients with FMF. Exp Biol Med 233:1324–1333, 2008

Key words: ASC; amyloidosis; speck; microtubules

Introduction

The hereditary autoinflammatory diseases, including familial Mediterranean fever (FMF), cryopyrin-associated periodic syndromes (CAPS), tumor necrosis factor–associated periodic syndrome (TRAPS), and hyper-IgD syndrome are associated with mutations in several proteins now believed to be involved in innate immune pathways (1). These diseases are characterized by systemic inflammation with high acute phase inflammatory markers and especially high levels of serum amyloid A (SAA). Secondary amyloidosis is a serious and life-threatening complication for patients with FMF, TRAPS, and CAPS (2). In the case of hyper-IgD syndrome, amyloidosis is much less common but has been reported (3).

FMF is the most common of the autoinflammatory diseases and is characterized by recurrent attacks of fever with localized, painful inflammation. Attacks are often associated with high levels of acute phase reactants, and inflammation typically involves the peritoneum, pleura, joints, and skin (4). More than in any of the other periodic fever syndromes, FMF makes patients susceptible to multiorgan deposition of amyloid. In fact, before the use

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of colchicine as a prophylactic treatment for FMF, amyloidosis was observed in as many as 70% of patients with FMF older than 40 years, although the actual incidence was quite variable in different ethnic groups (5). Although the most serious impact of amyloidosis is seen in the kidneys, it can also affect the gastrointestinal tract, liver, spleen, heart, and testes. Significant amyloid deposits found especially in glomeruli, tubules, and interstitium in the kidney causes nephrotic syndrome and chronic renal failure, which may be fatal.

In the case of FMF, but not in the other autoinflammatory diseases, the advent of colchicine treatment has had a remarkable effect on disease progression and outcome (6, 7). Colchicine use not only reduces the frequency and severity of FMF attacks but also prevents the development of amyloidosis. Even in the nephrotic stage of this disease, colchicine usage may reduce kidney damage.

The FMF gene, which is located on short arm of chromosome 16 and is symbolized as *MEFV* (Mediterranean fever), encodes a protein called pyrin or marenostrin (8, 9). Most FMF-causing pyrin mutations are found in the C-terminal SPRY domain, including the most common mutation M694V. This mutation is of particular interest, because it correlates with a high incidence of amyloidosis. Although the function of pyrin is not clearly understood, it appears to play an important role in the inflammatory pathways that characterize the innate immune system. At least part of this function is mediated through pyrin's interactions with the apoptotic speck protein ASC (10), a 195–amino acid adaptor protein that contains an N-terminal pyrin domain (PyD) and a C-terminal caspase recruitment domain (CARD). These two domains are related structures of the death domain family that act as protein-protein interaction interfaces. Through these two domains, ASC acts as an agglutinating platform for a wide variety of proteins involved in NF-κB activation, apoptosis, and IL-1β processing (11). The multiprotein ASC-dependent platform associated with IL-1β processing has been termed the inflammasome (12). Several different types of inflammasome have been documented, most of which contain ASC and caspase-1 as well as a variety of accessory proteins, including NALP1, NALP2, NALP3 (or cryopyrin), and pyrin (13–17). ASC also forms a death-associated platform that has been termed a pyroptosome because of its role in rapid caspase-1–mediated cell death (18).

On a cellular level, ASC can be found in two general patterns: either distributed throughout the cell (in the nucleus and cytoplasm) or concentrated in a perinuclear aggregate that was initially called a "speck" (10, 19). The exact function of distributed ASC remains to be determined, but speck formation plays a role in caspase-1 activation, IL-1β processing, and cell death (10, 18, 19).

In myeloid cells, ASC specks that form in response to potassium efflux recruit caspase-1. Agglutination of caspase-1 causes rapid activation of this protease, causing cell death within minutes after speck formation. This process, which is caspase-1–dependent, is called pyroptosis, and the speck in this case has been termed a pyroptosome (18).

In a variety of nonmyeloid cultured cells transfected with ASC, specks form within 6 hrs of transfection, and speck formation is associated with cell death (10, 19). However, because these cells lack caspase-1, the mechanism underlying cell death must be different than that in myeloid cells. These differences are important because ASC is expressed in a variety of nonmyeloid cells (20).

By its direct interaction with ASC, pyrin has the potential to modulate the ASC death machinery. Indeed, speck formation is accelerated in the presence of pyrin (10). Cells transfected with ASC alone form specks at a rate of 3% to 4%, whereas cells co-transfected with ASC and pyrin form specks at a rate of 20% to 22% (10). Despite this acceleration, speck-positive cells that also contain pyrin succumb more slowly to death (10). How (or whether) the ASC-pyrin interaction fits into the clinical picture of FMF inflammatory attacks or explains the predisposition of patients with FMF to amyloidosis is not yet clear.

Given the ability of pyrin to modulate ASC speck formation and subsequent apoptosis and given the known beneficial effects of colchicine in preventing FMF attacks, the initial aims of this study were to determine the effect of FMF-causing mutations in pyrin, particularly the M694V mutation, on speck formation and to investigate whether speck assembly requires an intact microtubular network. We also explored the fate of the large ASC speck-like structures after cell demise. Finally, we tested whether the expression or distribution of ASC is altered in kidneys of patients with FMF and amyloid deposition. The results of these experiments lead us to posit that ASC specks may be tied to amyloidosis and suggest a potential mechanism underlying the susceptibility of patients with FMF to amyloid deposition and a possible explanation for the efficacy of colchicine in treating amyloidosis.

Materials and Methods

Cell Culture and Plasmids. HeLa and COS-7 cells were grown in Dulbecco's modified Eagle medium plus 10% fetal bovine serum. For experiments, cells were plated onto glass coverslips placed in six-well culture plates. Cells were transfected by using the FuGENE-6 transfection reagent (Roche Applied Science, Indianapolis, IN). Myc-ASC, myc-pyrin, and myc-M694V pyrin were expressed by using the pCMV-Tag3A expression vector (Stratagene, La Jolla, CA). ASC-FLAG was generated by using the pCMV-Tag2A vector (Stratagene). ASC-YFP was generated by using the pE-YFP vector from Clontech (Mountain View, CA).

ASC Speck Assay and Nocodazole Application. The rate of speck formation was examined in HeLa cells transfected with ASC-FLAG with either empty vector (pcDNA3.1), myc-pyrin, or myc-M694V pyrin. Six hours after transfection, vehicle (dimethylsulfoxide [DMSO]) or

4.15 μ*M* nocodazole was added to the cells, and the cultures were placed on ice for 10 mins to depolymerize microtubules. The cells were then returned to 37°C and fixed 24 hrs later by using 4% paraformaldehyde in phosphate-buffered saline (PBS). The percentage of speck-positive cells was calculated as the number of specks divided by the total number of transfected cells.

Statistical Analysis. Statistical analysis was performed by using GraphPad Prism software. *P* values were calculated by using an unpaired two-tailed *t* test, and *P* values <0.05 were considered to be significant.

Antibodies. Mouse-α-FLAG (M2 monoclonal), mouse α-tubulin (monoclonal), and rabbit α-myc (polyclonal) antibodies were obtained from Sigma (St. Louis, MO). Goat α-mouse AF488 and goat α-rabbit AF568 fluorescent secondary antibodies were purchased from Molecular Probes (Invitrogen, Eugene, OR). The ASC mouse monoclonal antibody was kindly provided by Dr. Junya Masumoto (Shinshu University, School of Medicine, Nagano, Japan).

Immunofluorescence. Cells were fixed for 30 mins in 4% paraformaldehyde in PBS, permeabilized by using 0.2% Triton X-100 in PBS, and blocked by using 0.1% Tween-20 in PBS containing 10% goat serum and 0.01 g/mL bovine serum albumin (BSA). Primary and secondary antibody staining were carried out as described in the text, and nuclei were counterstained with DAPI. Cells were visualized by using a Nikon E2000 inverted fluorescent microscope, a Nikon E800 fluorescent microscope, or a Zeiss LSM 510 confocal microscope.

Patients. Patients were followed at the Hacettepe University, Faculty of Medicine, Ihsan Dogramaci Children's Hospital, Pediatric Nephrology and Rheumatology Unit in Ankara, Turkey. Fifteen patients with amyloid-positive FMF and 10 control cases (five biopsies taken from patients with a diagnosis other than FMF and five with focal segmental glomerular sclerosis [FGS]) were included in this study. Informed consent was obtained from patients. Five patients with FMF eligible for the mutation analysis were tested for *MEFV* mutations, and all were homozygous for the M694V mutation. Biopsies of the other 10 patients did not yield sufficient material for mutation analysis.

Immunohistochemical Staining of ASC. Immunohistochemical staining was performed with the anti-ASC monoclonal antibody by using the streptavidin-biotin-peroxidase method (DAKO LSAB Kit, Dako, Carpenteria, CA). Culture fluids of the hybridoma producing the anti-ASC monoclonal antibody were used as the primary antibody, and Tris-buffered saline (TBS) was used in place of the primary antibody as the negative control. Background staining in the sections was minimal. Five-micrometer-thick sections of formalin-fixed, paraffin-embedded tissues were cut and deparaffinized. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide in methanol for 30 mins, and then the sections were washed and blocked with 1% BSA in TBS for 1 hr. Sections were incubated with purified primary antibodies overnight at 4°C and then with biotinized anti-mouse secondary antibody for 60 mins at room temperature. After washing threetimes with TBS, sections were incubated with horseradish peroxidase–conjugated streptavidin for 30 mins. After washing three times with TBS, the staining reaction was developed with 3,3-diaminobenzidine (Sigma Chemical, Cambridge, UK). Counterstaining was performed with hematoxylin. Sections were visualized by using an Olympus BX51 light microscope.

Congo Red Staining. Five-micrometer-thick sections of formalin-fixed, paraffin-embedded renal tissues were stained in a congo red working solution for 10 mins, rinsed in distilled water, and differentiated quickly (5–10 dips) in an alkaline alcohol solution. Sections were rinsed in tap water and counterstained with hematoxylin. Orange to red staining deposits that showed apple-green birefringence with polarizing light were accepted as amyloid deposition.

Video Recordings of ASC Speck Formation. HeLa cells were transfected with ASC-YFP and allowed to form specks for 24 hrs. Using phase contrast optics, we selected a field containing speck-positive cells as well as nontransfected cells. A fluorescence image was obtained of this field to document the location of the speck-containing cells. The cells were then maintained at 37°C under the microscope for 24 hrs, and phase contrast images were taken every 5 mins. The complete image stack was assembled by using NIH Image and converted to QuickTime movies. All cells were numbered, and the fate of each cell was recorded. The procedure was repeated seven times.

Results

Delayed Death of Nonmyeloid Cells Transfected with ASC. Previous work by our laboratory (10) and others (19) established an association between the presence of ASC specks and increased cell death on a population level. Fernandes-Alnemri *et al*. (18) were the first to examine this process on a single-cell basis in a myeloid cell line (HL-60). Those studies revealed rapid formation of the speck (pyroptosome) within 3 mins and almost immediate cell death (18). However, our earlier studies of nonmyeloid cells suggested that cell death was not an immediate consequence of speck formation (10). To examine this further, we used time-lapse video microscopy to follow the death of transfected HeLa cells containing ASC specks. Cells were transfected with ASC-YFP, and 24 hrs after transfection fluorescent images were captured to identify cells that contained specks. Once a field of speck-positive cells was identified, phase contrast images were captured every 5 mins for 24 hrs to determine cell fate.

Figure 1A shows a typical field of transfected and nontransfected cells under phase contrast optics. A fluorescence image obtained of this same field is provided in Figure 1B. Several frames extracted from the ensuing movie are shown in Figure 1C through F. Here eight cells were

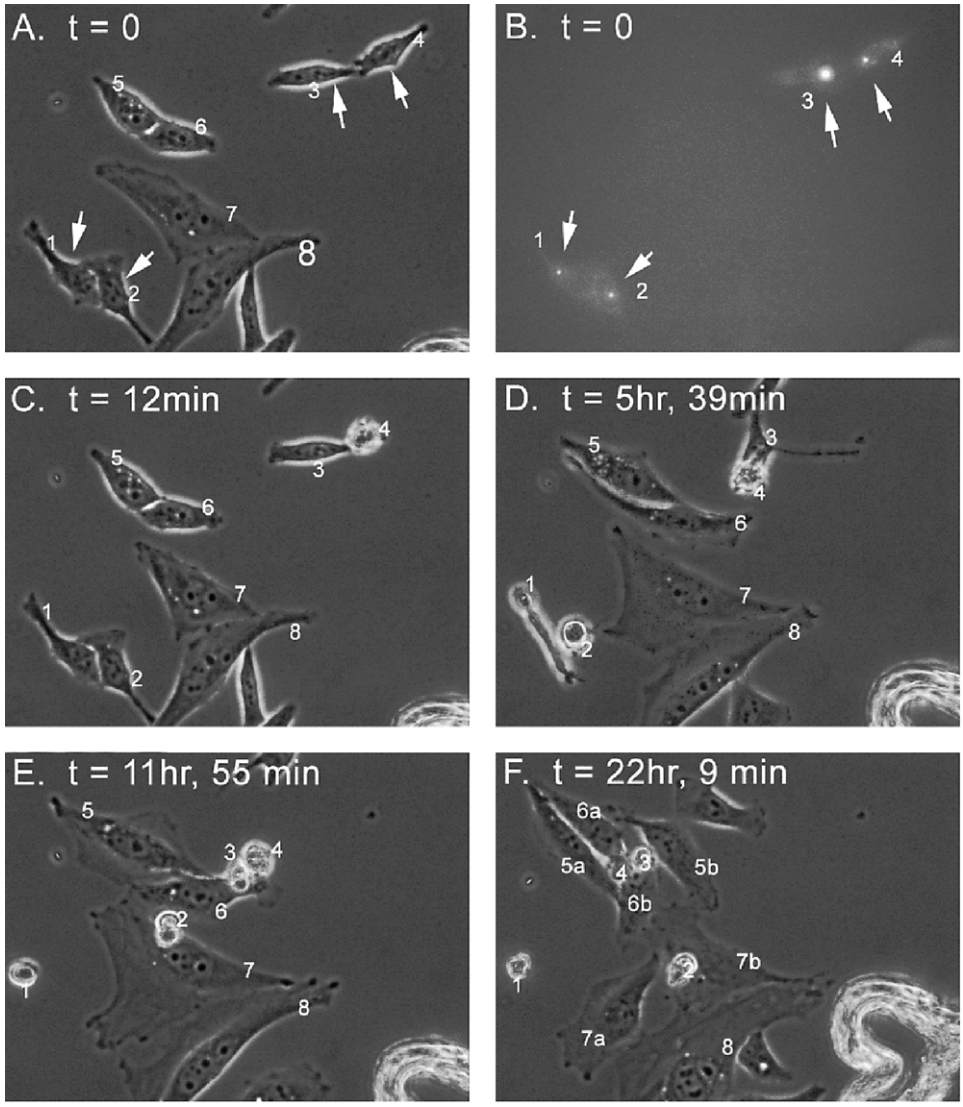


Figure 1. The presence of specks presages cell death. HeLa cells were transfected with ASC-YFP and imaged for 24 hrs as described in the text. Each cell was given a number, and the fate of the cells was followed with time. (A) Phase contrast image of the starting field. (B) Fluorescence image of the starting field. (C–F) Shown are panels extracted from the movie at the indicated times. All four cells with specks (#1–4) underwent cell death during the 24 hrs of observation. None of the nontransfected cells (#5–8) died, but three of these divided (#5–7).

tracked: four had specks (#1–4), and four did not (#5–8). The first of the speck-containing cells to die (#4) succumbed in the first 12 mins of observation (Fig. 1C). By 5.5 hrs, another cell (#2) had died, and two other cells (#1 and #3) showed signs of imminent death (Fig. 1D). By nearly 12 hrs, all four speck-containing cells had died (Fig. 1E). Continued observation revealed that cells #5 through 7 underwent mitosis, whereas cell #8 neither died nor divided (Fig. 1F). None of the four nontransfected cells died.

These data demonstrate, on an individual cell basis, that once a speck forms, the speck-containing cell is destined for death, but death is not immediate. Analysis of seven independent experiments revealed that cells containing specks died at a rate of 95.1%, whereas cells without specks died at a rate of 11.4%. Cell death occurred as early as 12 mins and as late as 22 hrs after imaging was initiated. Many of the nontransfected cells completed mitosis, a finding indicating that the observation conditions were not the cause of death in speck-containing cells. Thus, the death of nonmyeloid cells (which lack caspase-1) after speck formation is delayed for as long as several hours. As shown in our previous work, the presence of pyrin may further delay this cell death process (10).

ASC Specks Are Cytosolic and Perinuclear. The time-lapse videos revealed that ASC specks are most often located very near the nucleus; some appeared to be either in or on top of the nucleus. To further clarify whether specks are located in the cytoplasm or nucleus, we used confocal microscopy. Figure 2B contains a tracing of the signal from the cell in Figure 2A that contained an ASC speck. The vector of the tracing is shown by the red line in Figure 2A. The speck was labeled with AlexaFluor568-

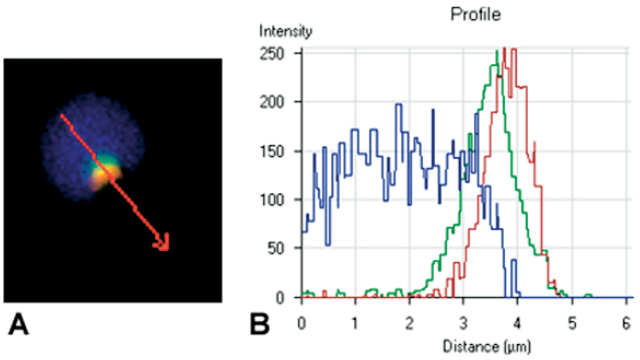


Figure 2. ASC specks are cytosolic. To further clarify the subcellular localization of the speck, we used confocal microscopy. (A) The speck is labeled with AlexaFluor568-conjugated anti-FLAG to detect FLAG-tagged ASC, whereas the nucleus is labeled with DAPI. (B) Shown is a tracing of the signal from the cell in panel A that contains an ASC speck; clearly the speck is cytosolic. The vector of the tracing is shown by the red line in panel A. A color version of this figure is available in the online journal.

conjugated anti-FLAG to detect FLAG-tagged ASC, whereas the nucleus was labeled with DAPI. The tracing clearly shows that the speck was outside the nucleus. This finding was true of at least 20 specks that were imaged in this way. The co-transfection of either wild-type or mutant forms of pyrin with ASC did not change the location of ASC specks (data not shown).

ASC Specks Are Located near the Microtubule-Organizing Center (MTOC) of Apoptotic Cells. Because the preceding data indicated that specks are perinuclear, we next tested whether specks co-localize with the MTOC, a structure that is also perinuclear. To investigate this hypothesis, COS-7 cells were transfected with ASC-myc, and after 24 hrs, cells were costained for myc and tubulin. Random fields of fixed and stained cells were imaged, and the number of specks near the MTOC were counted and compared with the total number of specks. Using this method, we calculated that specks localized near the MTOC at a rate of 89.6%, much higher than random localization would indicate (Fig. 3).

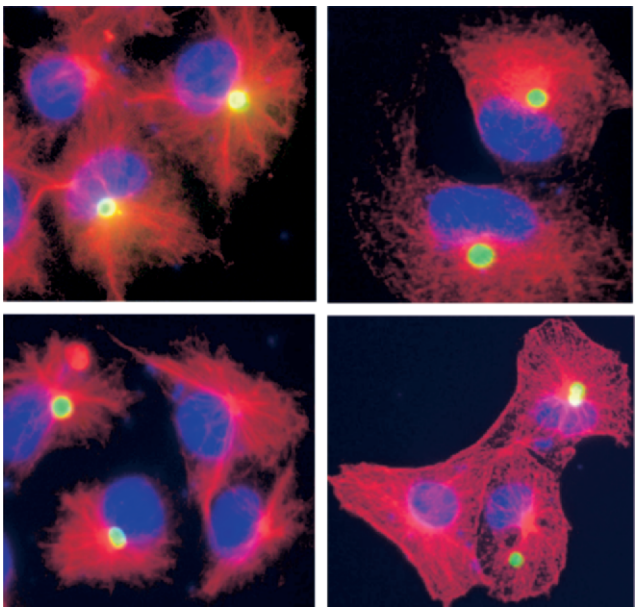
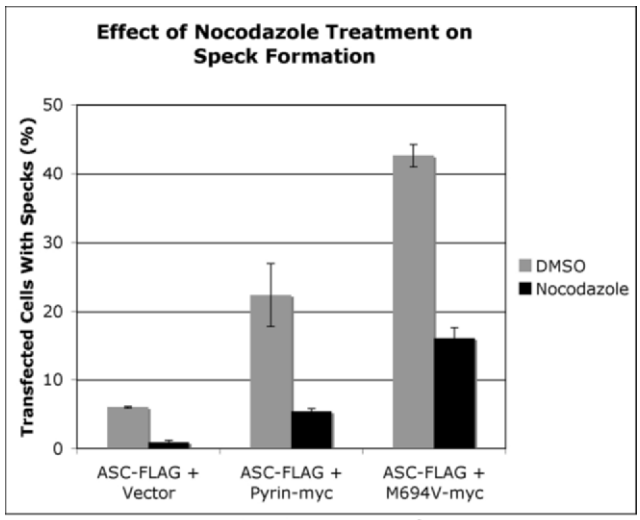


Figure 3. Specks co-localize with the MTOC. COS-7 cells were transfected with myc-tagged ASC and then stained with both an anti-myc antibody to detect ASC (red) and an anti-tubulin antibody to identify microtubules (green). Nuclei were counterstained with DAPI. Yellow color indicates overlap between green and red staining. We found that 89.6% of the ASC specks co-localized with the MTOC. Examples of four fields are shown. A color version of this figure is available in the online journal.



Transfection 1	Treatment 1	Transfection 2	Treatment 2	p Value
ASC-FLAG	DMSO	ASC-FLAG + Pyrin-myc	DMSO	p=0.049
ASC-FLAG	DMSO	ASC-FLAG + M694V-myc	DMSO	p<0.0001
ASC-FLAG + Pyrin-myc	DMSO	ASC-FLAG + M694V-myc	DMSO	p=0.0003
ASC-FLAG	DMSO	ASC-FLAG	Nocodazole	p<0.0001
ASC-FLAG + Pyrin-myc	DMSO	ASC-FLAG + Pyrin-myc	Nocodazole	p=0.0041
ASC-FLAG + M694V-myc	DMSO	ASC-FLAG + M694V-myc	Nocodazole	p<0.0001

Figure 4. Nocodazole reduces the rate of speck formation. HeLa cells were transfected with ASC-FLAG and then treated with DMSO or nocodazole. The addition of nocodazole reduced speck formation from 6% to 0.9% ($P < 0.0001$). Whereas co-transfection of pyrin with ASC increased speck formation to 22% ($P = 0.049$), nocodazole reduced this rate to 5% ($P = 0.0041$). Co-transfection of M694V pyrin further increased the rate of speck formation to 43% ($P = 0.0003$), and nocodazole reduced this rate to 16% ($P < 0.0001$).

Microtubule Disruption Reduces Speck Formation. The consistent colocalization of ASC specks with the MTOC suggested that the transition from distributed, soluble ASC to aggregated specks may depend on an intact tubulin cytoskeleton. To test this possibility, we exposed cells to nocodazole, a microtubule toxin with effects similar to those of colchicine. HeLa cells were transfected with ASC-FLAG and treated with either nocodazole or vehicle (DMSO). When cells were treated with DMSO after transfection with ASC-FLAG, 6% of transfected cells formed specks within 24 hrs, a result consistent with that of previous control experiments without DMSO (10). However, after treatment with nocodazole, significantly fewer cells contained specks; 0.9% of transfected cells were speck-positive after 24 hrs ($P < 0.0001$; Fig. 4).

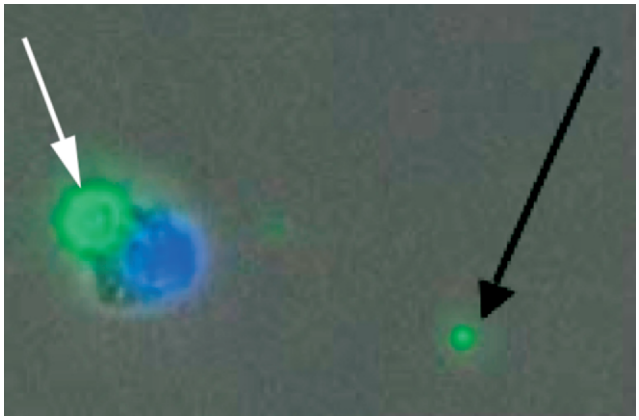


Figure 5. Specks remain stable in the extracellular space. Cultures containing COS-7 cells that overexpressed ASC contained stable extracellular specks (black arrow). Some dying cells appeared to be expelling the specks into the extracellular space (white arrow). A color version of this figure is available in the online journal.

Our earlier studies indicated that the presence of pyrin increases the rate of speck formation (10). Therefore, we tested whether nocodazole also reduces the rate of speck formation in cells co-transfected with ASC and pyrin. In addition, we tested the effect of mutant pyrin on speck formation in the presence or absence of nocodazole. As shown in Figure 4, in the absence of nocodazole, co-transfection of cells with wild-type pyrin and ASC resulted in increased speck formation as previously documented ($P = 0.049$; see Ref. 10). Interestingly, a further increase in speck number was seen when mutant pyrin was co-transfected with ASC ($P = 0.003$). In the presence of nocodazole, however, the number of specks was significantly reduced. Nocodazole reduced speck number by more than 6-fold in the absence of pyrin ($P < 0.0001$), by more than 4-fold in the presence of pyrin ($P = 0.0041$), and by more than 2.5-fold in the presence of mutant pyrin ($P < 0.0001$). The somewhat attenuated effect of nocodazole in the pyrin-transfected and mutant pyrin–transfected cells may reflect the fact that in the presence of pyrin, more specks were formed in the 6 hrs before the administration of nocodazole. Indeed, when we lengthened the pretreatment time to 16 hrs, the effect of nocodazole was even further attenuated by the presence of pyrin (data not shown). Together, these results demonstrate that (i) speck formation is increased by wild-type pyrin and even more so by mutant pyrin and (ii) speck formation is facilitated by an intact microtubular network.

ASC Specks Are Stable in Extracellular Space. In the course of these experiments, we occasionally noted specks that appeared to be outside of cells (Fig. 5). In addition, some dying cells appeared to extrude a speck. Such specks were stable in the extracellular space of the culture dish for prolonged periods of time (the longest examination period was 72 hrs). Although we have not confirmed that such "bare" specks also survive *in vivo*, the stability of these structures *in vitro* was remarkable.

ASC Is Aberrantly Expressed in Glomeruli of Patients with FMF and Amyloid. The expression of Pycard, the gene for ASC, is induced under inflammatory conditions (21). Because patients with FMF often experience kidney disease and because colchicine prevents kidney disease in these patients and modulates ASC speck formation, we tested whether ASC expression is associated with kidney disease in patients with FMF. We examined renal tissues obtained by biopsy from patients who had FMF and were homozygous for the M694V mutation and from control patients (those with a diagnosis other than FMF) after staining with an anti-ASC antibody obtained from Dr. Junya Masumoto (Shinshu University, Nagano, Japan). In the control group, we also included five patients with FGS (FGS is associated with apoptosis of glomerular cells). In all control tissues obtained by biopsy, ASC was detectable only in renal tubules. No ASC expression was seen in glomeruli of any of the control patients (Fig. 6A). However, in renal tissues obtained by biopsy from patients with amyloid-positive FMF, ASC was detected in renal tubules and in glomeruli (Fig. 6B).

To investigate whether ASC expression correlated with amyloid deposition, renal tissue obtained by biopsy from patients with FMF were analyzed after congo red staining. Sequential sections were stained to determine the localization of ASC. In all 15 patients, there was a direct correlation between ASC expression and the presence of amyloid deposits; that is, ASC expression was detected in every glomerulus containing amyloid deposits (Fig. 6C and D). Generally, ASC expression was high in the region immediately surrounding the amyloid deposits (especially on the boundaries) but much lower inside the deposits.

Discussion

In this study, we further characterized ASC speck formation in nonmyeloid cells and examined ASC expression in the kidneys of patients with FMF. Earlier work showed that ASC is induced by inflammatory cytokines (19, 21), but the differential activation of ASC in glomeruli of patients with FMF as shown here has not been previously noted. The coexistence of ASC in every single glomerulus with visible amyloid was a striking finding of our study. In addition, the distribution of ASC expression in relation to the amyloid deposits within a given glomerulus is interesting: the highest concentrations of ASC were found in cells bordering the amyloid deposits. There are two possible reasons for this, one trivial and one potentially instructive. First, amyloid deposits may simply mask ASC epitopes and may not allow the simultaneous visualization of ASC in the presence of concentrated amyloid. Alternatively, it is also possible that in the areas where amyloid is most prevalent, cellular ASC is no longer distributed throughout the cell but has already been collected into specks. Although ASC specks are readily visible in flat, well-spread cells under culture conditions, it is more difficult to detect them *in situ* in a tissue section. We did, in a few instances, identify

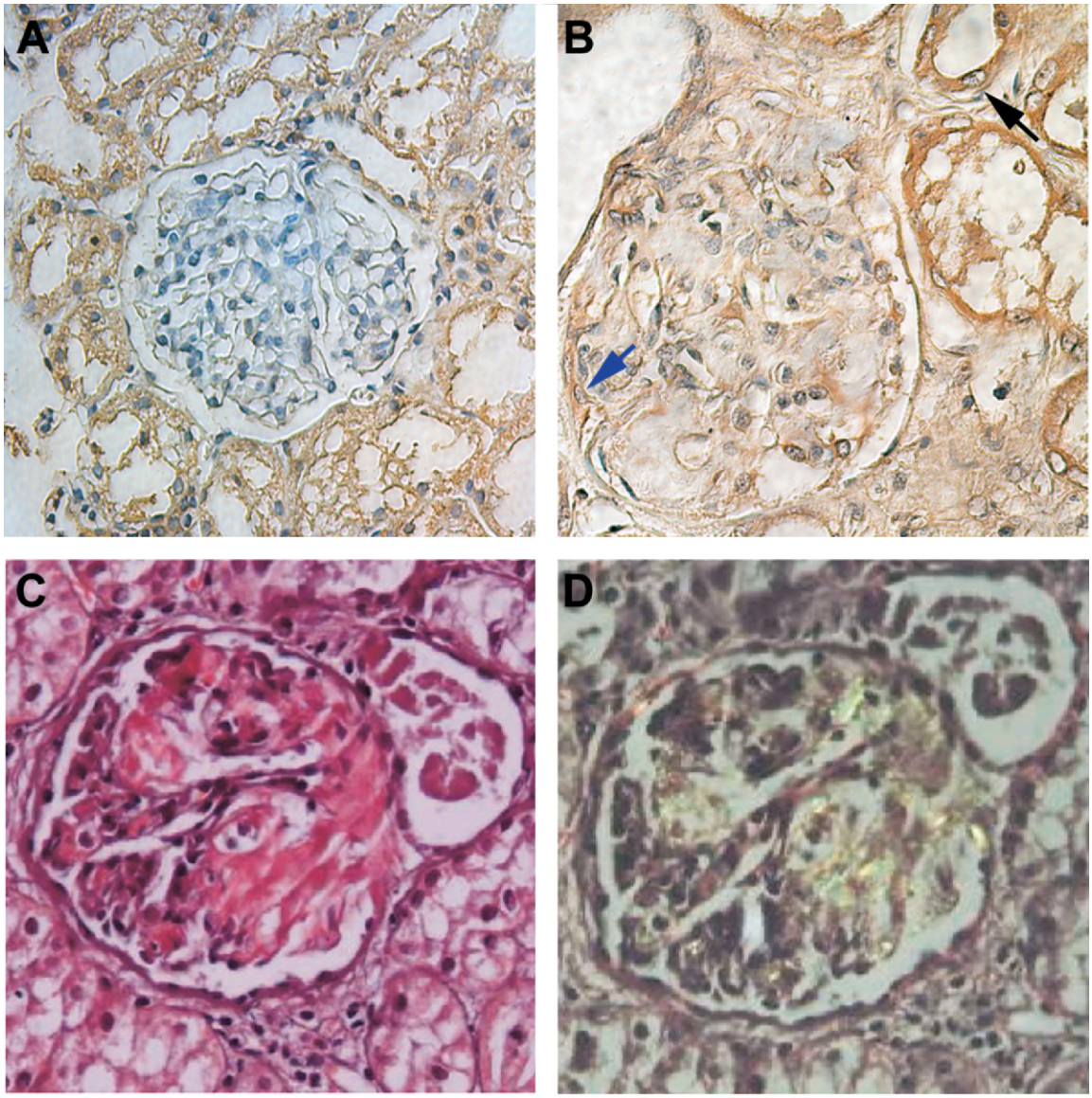


Figure 6. ASC is expressed in the glomeruli of patients with FMF and amyloidosis. (A) Immunostaining of a control kidney showed that ASC is expressed in renal tubules only. (B) In patients with FMF and amyloid, ASC was expressed in tubules (black arrow) and in glomeruli (blue arrow). (C and D) Birefringence of congo red staining under polarized light showed clear amyloid deposition in the FMF kidney. ASC appeared to be expressed at the boundaries of amyloid deposition. A color version of this figure is available in the online journal.

speck-like structures within the amyloid-containing regions, but it was impossible to say with certainty that these were ASC specks.

ASC expression was never detected in glomeruli of patients who did not have FMF, although patients with other types of autoinflammatory diseases were not examined. To rule out the possibility that glomerular cell apoptosis alone might be sufficient to promote amyloid deposition, we included five patients with a diagnosis of FGS, a disease characterized by sclerosis in glomerular tufts that is distributed in a segmental manner (22). Glomerular cell apoptosis is believed to be an important event in the progression of this disease (23). The fact that none of the five patients with FGS exhibited glomerular amyloid deposition or glomerular expression of ASC leads us to conclude that the process of glomerular apoptosis itself is not a trigger for glomerular amyloidosis or for ASC induction. This further supports our hypothesis that a more specific functional connection may exist between ASC expression and amyloid deposition in glomeruli of patients with FMF.

Patients with FMF are particularly prone to amyloidosis; before the advent of colchicine, this complication was responsible for significant mortality of patients with FMF (5). A variant of FMF, type II FMF, has been documented in which amyloid deposition and resulting uremia precedes the more typical inflammatory attacks (2). It appears, however, that the connection between mutant forms of pyrin and

amyloid deposition is not a simple direct one, because not all patients with FMF develop this complication and the extent of amyloidosis can vary markedly among members of the same family carrying the same mutation. Moreover, clear environmental effects have been documented; for example, the rate of amyloidosis in Armenians living in the United States (almost nil) is vastly different than that of Armenians in their native country (24%; Ref. 24). Some factors that appear to predispose to amyloidosis have been identified: the M694V mutation has been linked to severe disease and an increased risk of amyloidosis in some studies (25–32) but not in others (33–35); the SAA1 gene haplotype is associated with up to 7-fold increased risk of amyloidosis (28, 30, 33, 36), and male patients have as much as a 4-fold greater risk of amyloid deposition than do female patients (28, 30, 37). Although it is clear that amyloidosis is a common complication of FMF, it is also obvious that a multifactorial mechanism is at play.

The process of amyloid deposition has been studied in cell culture and in mouse models and kinetic models have been proposed (38). The results of these studies have suggested a biphasic mechanism in which a long lag period (predicting a nucleation step) is followed by a rapid deposition phase. Stabilization of formed amyloid fibrils is also an important part of the kinetics of amyloid accumulation. The initial lag period before nucleation can be shortened in experimental systems by adding a poorly characterized glycerol extract of spleen from an amyloidotic animal (39). This material is known as amyloid accelerating factor (AEF); it has been proposed that AEF may act as a seed around which amyloid polymerizes (40). The key structural component of AEF is thought to be its β-sheet content (41). In this regard, it is interesting to note that both domains of ASC (the CARD and the PyD) are composed of six α-helical bundles, and there is little, if any, β sheet in the ASC molecule (42). However, the C-terminal PRYSPRY domain of pyrin is composed of multistranded β sheets, arranged in a β sandwich (43–45). This structure has been described as highly similar to that of the pea lectin (43). Interestingly, the structure of human serum amyloid P component (SAP), a protein that binds to all forms of amyloid and is present in all amyloid deposits, has also been likened to that of the pea lectin (46). Indeed, the overall structure of SAP closely resembles that of pyrin's PRYSPRY domain (compare Fig. 3 in Ref. 43 to Fig. 2 in Ref. 46). SAP is not required for amyloid deposition *in vitro*, but in SAP-deficient animals, amyloid deposition is slowed (47). SAP has also been implicated in the stabilization of amyloid fibrils *in vivo* (48). The striking structural similarities between SAP and the pyrin PRYSPRY domain are particularly interesting given the fact that more than 65% of the FMF-associated pyrin mutations are found in the PRYSPRY domain (INFEVERS database). If PRYSPRY does indeed associate with amyloid in a way similar to that of SAP, then pyrin mutations could have the potential to alter some aspect of amyloid formation-degradation kinetics.

If pyrin and ASC are together responsible for amyloid deposition, which cells of the glomerulus might express both proteins? There are at least three possible answers to this question. First, infiltrating inflammatory cells (*e.g.*, monocytes, neutrophils) are known to express ASC and pyrin. Second, recent studies indicate that after glomerular injury, bone marrow stem cells can give rise to mesangial cells of the glomeruli; as many as 12% of the glomerular cells were found to be bone marrow-derived after induction of experimental glomerulonephritis (49). Third, pyrin is expressed in a variety of fibroblastic cells (50, 51). In the kidney, myofibroblasts, which can be derived from mesangial cells or vascular cells, play a role in wound healing and disappear by apoptosis after the injury is resolved (52).

The data presented in this manuscript predict that in the presence of mutant forms of pyrin ASC specks form more readily in a cell that expresses both proteins. Furthermore, we showed that ASC specks can outlive the apoptotic cells and survive in the extracellular environment. We propose therefore that the presence of a large number of extracellular pyrin-coated specks could promote amyloid deposition in patients with FMF. Other unknown factors may modulate ASC expression, speck formation, speck extrusion, speck stability, or aspects of amyloid deposition and stability, accounting for the well-known variability in amyloid deposition among patients with FMF.

The association between ASC specks and the MTOC and the requirement for an intact microtubular system for efficient speck formation represent two additional important findings of our studies. Given these findings, it is interesting that data from several laboratories indicate that colchicine inhibits amyloid formation in a variety of experimental systems (53, 54). Although the other studies used different amyloid formation protocols, some have concluded that colchicine inhibits the nucleating step (53, 54), whereas others have suggested that the growth phase is inhibited (55–57). Our work indicates that the disruption of the microtubule system by nocodazole dramatically reduces speck nucleation. These data, together with the preceding ASC expression data, suggest the hypothesis that ASC specks (perhaps in association with pyrin) may promote amyloidogenesis inpatients with FMF. Further direct evidence using amyloid-forming systems *in vivo* and *in vitro* is required to lend further support to this hypothesis.

Patients with FMF are particularly prone to amyloidosis, but amyloid is also seen in patients with CAPS and TRAPS, two of the other so-called autoinflammatory diseases. However, colchicine treatment is not effective for CAPS and TRAPS (58). A possible explanation for this therapeutic difference might be that the promotion of amyloid in patients with FMF is a consequence of the presence of mutant pyrin on agglutinated ASC rather than a consequence of agglutinated ASC alone. It is also possible

that other aspects of the molecular composition of amyloid deposits themselves differ in FMF, CAPS, and TRAPS. This hypothesis remains to be studied. In the case of CAPS, it is interesting that cryopyrin shares with pyrin the ability to interact with ASC, and, in fact, this interaction is the basis for the generation of at least one form of inflammasome, a multiprotein complex that contains caspase-1 and can efficiently process IL-1β (12). Whether cryopyrin can also promote speck formation and whether speck-associated cryopyrin can survive in the extracellular space and play a role in the induction of amyloid remains to be studied.

In summary, the data presented here reveal a potentially important connection between mutant forms of pyrin, ASC expression, and amyloidosis in patients with FMF. Further functional studies are required to more completely explore this tantalizing link. These findings not only might explain the high incidence of amyloidosis in patients with untreated FMF but also could suggest a mechanism at the cellular level for the remarkable efficacy of colchicine in preventing and even reversing amyloidosis, specifically in these patients.

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1. Werts C, Girardin SE, Philpott DJ. TIR, CARD and PYRIN: three domains for an antimicrobial triad. Cell Death Differ 13:798–815, 2006.
2. van der Hilst JC, Simon A, Drenth JP. Hereditary periodic fever and reactive amyloidosis. Clin Exp Med 5:87–98, 2005.
3. van der Hilst JC, Simon A, Drenth JP. Molecular mechanisms of amyloidosis. N Engl J Med 349:1872–1873; author reply 1872–1873, 2003.
4. Aringer M. Periodic fever syndromes—a clinical overview. Acta Med Austriaca 31:8–12, 2004.
5. Gafni J, Ravid M, Sohar E. The role of amyloidosis in familial mediterranean fever. A population study. Isr J Med Sci 4:995–999, 1968.
6. Lidar M, Scherrmann JM, Shinar Y, Chetrit A, Niel E, Gershoni-Baruch R, Langevitz P, Livneh A. Colchicine nonresponsiveness in familial Mediterranean fever: clinical, genetic, pharmacokinetic, and socioeconomic characterization. Semin Arthritis Rheum 33:273–282, 2004.
7. Zemer D, Pras M, Sohar E, Modan M, Cabili S, Gafni J. Colchicine in the prevention and treatment of the amyloidosis of familial Mediterranean fever. N Engl J Med 314:1001–1005, 1986.
8. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. The International FMF Consortium. Cell 90:797–807, 1997.
9. A candidate gene for familial Mediterranean fever. The French FMF Consortium. Nat Genet 17:25–31, 1997.
10. Richards N, Schaner P, Diaz A, Stuckey J, Shelden E, Wadhwa A, Gumucio DL. Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. J Biol Chem 276:39320–39329, 2001.
11. Gumucio DL, Diaz A, Schaner P, Richards N, Babcock C, Schaller M, Cesena T. Fire and ICE: the role of pyrin domain-containing proteins in inflammation and apoptosis. Clin Exp Rheumatol 20(Suppl 4):S45–S53, 2002.
12. Mariathasan S. ASC, Ipaf and Cryopyrin/Nalp3: bona fide intracellular adapters of the caspase-1 inflammasome. Microbes Infect 9:664–671, 2007.
13. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity 20:319–325, 2004.
14. Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T, Alnemri ES. Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. J Biol Chem 276:28309–28313, 2001.
15. Yu JW, Wu J, Zhang Z, Datta P, Ibrahimi I, Taniguchi S, Sagara J, Fernandes-Alnemri T, Alnemri ES. Cryopyrin and pyrin activate caspase-1, but not NF-κB, via ASC oligomerization. Cell Death Differ 13:236–249, 2006.
16. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. Mol Cell 10:417–426, 2002.
17. Srinivasula SM, Poyet JL, Razmara M, Datta P, Zhang Z, Alnemri ES. The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. J Biol Chem 277:21119–21122, 2002.
18. Fernandes-Alnemri T, Wu J, Yu JW, Datta P, Miller B, Jankowski W, Rosenberg S, Zhang J, Alnemri ES. The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ 14:1590–1604, 2007.
19. Masumoto J, Taniguchi S, Ayukawa K, Sarvotham H, Kishino T, Niikawa N, Hidaka E, Katsuyama T, Higuchi T, Sagara J. ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. J Biol Chem 274:33835–33838, 1999.
20. Masumoto J, Taniguchi S, Nakayama K, Ayukawa K, Sagara J. Murine ortholog of ASC, a CARD-containing protein, self-associates and exhibits restricted distribution in developing mouse embryos. Exp Cell Res 262:128–133, 2001.
21. Shiohara M, Taniguchi S, Masumoto J, Yasui K, Koike K, Komiyama A, Sagara J. ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils. Biochem Biophys Res Commun 293:1314–1318, 2002.
22. Kanjanabuch T, Lewsuwan S, Kitiyakara C, Cheunsuchon B, Eiam-Ong S. Update in pathophysiology and histopathology of focal segmental glomerulosclerosis. J Med Assoc Thai 89(Suppl 2):S262–S279, 2006.
23. Chihara Y, Ono H, Ishimitsu T, Ono Y, Ishikawa K, Rakugi H, Ogihara T, Matsuoka H. Roles of TGF-beta1 and apoptosis in the progression of glomerulosclerosis in human IgA nephropathy. Clin Nephrol 65:385–392, 2006.
24. Schwabe AD, Peters RS. Familial Mediterranean fever in Armenians. Analysis of 100 cases. Medicine (Baltimore) 53:453–462, 1974.
25. Mimouni A, Magal N, Stoffman N, Shohat T, Minasian A, Krasnov M, Halpern GJ, Rotter JI, Fischel-Ghodsian N, Danon YL, Shohat M. Familial Mediterranean fever: effects of genotype and ethnicity on inflammatory attacks and amyloidosis. Pediatrics 105:E70, 2000.
26. Ben-Chetrit E, Backenroth R. Amyloidosis induced, end stage renal disease in patients with familial Mediterranean fever is highly associated with point mutations in the MEFV gene. Ann Rheum Dis 60:146–149, 2001.
27. Mansour I, Delague V, Cazeneuve C, Dode C, Chouery E, Pecheux C, Medlej-Hashim M, Salem N, El Zein L, Levan-Petit I, Lefranc G, Goossens M, Delpech M, Amselem S, Loiselet J, Grateau G, Megarbane A, Naman R. Familial Mediterranean fever in Lebanon: mutation spectrum, evidence for cases in Maronites, Greek Orthodoxes, Greek Catholics, Syriacs and Chiites and for an association between amyloidosis and M694V and M694I mutations. Eur J Hum Genet 9:51–55, 2001.
28. Gershoni-Baruch R, Brik R, Zacks N, Shinawi M, Lidar M, Livneh A.

The contribution of genotypes at the MEFV and SAA1 loci to amyloidosis and disease severity in patients with familial Mediterranean fever. Arthritis Rheum 48:1149–1155, 2003.

29. Langevitz P, Livneh A, Padeh S, Zaks N, Shinar Y, Zemer D, Pras E, Pras M. Familial Mediterranean fever: new aspects and prospects at the end of the millenium. Isr Med Assoc J 1:31–36, 1999.
30. Cazeneuve C, Ajrapetyan H, Papin S, Roudot-Thoraval F, Genevieve D, Mndjoyan E, Papazian M, Sarkisian A, Babloyan A, Boissier B, Duquesnoy P, Kouyoumdjian JC, Girodon-Boulandet E, Grateau G, Sarkisian T, Amselem S. Identification of MEFV-independent modifying genetic factors for familial Mediterranean fever. Am J Hum Genet 67:1136–1143, 2000.
31. Brik R, Shinawi M, Kepten I, Berant M, Gershoni-Baruch R. Familial Mediterranean fever: clinical and genetic characterization in a mixed pediatric population of Jewish and Arab patients. Pediatrics 103:e70, 1999.
32. Cazeneuve C, Sarkisian T, Pecheux C, Dervichian M, Nedelec B, Reinert P, Ayvazyan A, Kouyoumdjian JC, Ajrapetyan H, Delpech M, Goossens M, Dode C, Grateau G, Amselem S. MEFV-Gene analysis in Armenian patients with familial Mediterranean fever: diagnostic value and unfavorable renal prognosis of the M694V homozygous genotype-genetic and therapeutic implications. Am J Hum Genet 65:88–97, 1999.
33. Altiok O, Seguret F, Touitou I. MEFV sequence variants and amyloidosis: still an enigmatic question. Hum Mutat 21:96–97, 2003.
34. Akar E, Yalcinkaya F, Akar N. Is the Ala138Gly alteration of MEFV gene important for amyloidosis? Hum Mutat 17:71, 2001.
35. Yalcinkaya F, Cakar N, Misirlioglu M, Tumer N, Akar N, Tekin M, Tastan H, Kocak H, Ozkaya N, Elhan AH. Genotype-phenotype correlation in a large group of Turkish patients with familial Mediterranean fever: evidence for mutation-independent amyloidosis. Rheumatology (Oxford) 39:67–72, 2000.
36. Yilmaz E, Balci B, Kutlay S, Ozen S, Erturk S, Oner A, Besbas N, Bakkaloglu A. Analysis of the modifying effects of SAA1, SAA2 and TNF-α gene polymorphisms on development of amyloidosis in FMF patients. Turk J Pediatr 45:198–202, 2003.
37. Saatci U, Ozen S, Ozdemir S, Bakkaloglu A, Besbas N, Topaloglu R, Arslan S. Familial Mediterranean fever in children: report of a large series and discussion of the risk and prognostic factors of amyloidosis. Eur J Pediatr 156:619–623, 1997.
38. Murphy RM. Kinetics of amyloid formation and membrane interaction with amyloidogenic proteins. Biochim Biophys Acta 1768:1923–1934, 2007.
39. Axelrad MA, Kisilevsky R, Willmer J, Chen SJ, Skinner M. Further characterization of amyloid-enhancing factor. Lab Invest 47:139–146, 1982.
40. Magy N, Liepnieks JJ, Benson MD, Kluve-Beckerman B. Amyloid-enhancing factor mediates amyloid formation on fibroblasts via a nidus/template mechanism. Arthritis Rheum 48:1430–1437, 2003.
41. Kisilevsky R, Lemieux L, Boudreau L, Yang DS, Fraser P. New clothes for amyloid enhancing factor (AEF): silk as AEF. Amyloid 6:98–106, 1999.
42. Hofmann K. The modular nature of apoptotic signaling proteins. Cell Mol Life Sci 55:1113–1128, 1999.
43. Grutter C, Briand C, Capitani G, Mittl PR, Papin S, Tschopp J, Grutter MG. Structure of the PRYSPRY-domain: implications for auto-inflammatory diseases. FEBS Lett 580:99–106, 2006.
44. Masters SL, Yao S, Willson TA, Zhang JG, Palmer KR, Smith BJ, Babon JJ, Nicola NA, Norton RS, Nicholson SE. The SPRY domain of SSB-2 adopts a novel fold that presents conserved Par-4-binding residues. Nat Struct Mol Biol 13:77–84, 2006.
45. Woo JS, Imm JH, Min CK, Kim KJ, Cha SS, Oh BH. Structural and functional insights into the B30.2/SPRY domain. EMBO J 25:1353–1363, 2006.
46. Emsley J, White HE, O'Hara BP, Oliva G, Srinivasan N, Tickle IJ, Blundell TL, Pepys MB, Wood SP. Structure of pentameric human serum amyloid P component. Nature 367:338–345, 1994.
47. Tennent GA, Lovat LB, Pepys MB. Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis. Proc Natl Acad Sci U S A 92:4299–4303, 1995.
48. Hind CR, Collins PM, Renn D, Cook RB, Caspi D, Baltz ML, Pepys MB. Binding specificity of serum amyloid P component for the pyruvate acetal of galactose. J Exp Med 159:1058–1069, 1984.
49. Ito T, Yorioka N, Yamamoto M, Kataoka K, Yamakido M. Effect of glucose on intercellular junctions of cultured human peritoneal mesothelial cells. J Am Soc Nephrol 11:1969–1979, 2000.
50. Centola M, Wood G, Frucht DM, Galon J, Aringer M, Farrell C, Kingma DW, Horwitz ME, Mansfield E, Holland SM, O'Shea JJ, Rosenberg HF, Malech HL, Kastner DL. The gene for familial Mediterranean fever, MEFV, is expressed in early leukocyte development and is regulated in response to inflammatory mediators. Blood 95: 3223–3231, 2000.
51. Diaz A, Hu C, Kastner DL, Schaner P, Reginato AM, Richards N, Gumucio DL. Lipopolysaccharide-induced expression of multiple alternatively spliced MEFV transcripts in human synovial fibroblasts: a prominent splice isoform lacks the C-terminal domain that is highly mutated in familial Mediterranean fever. Arthritis Rheum 50:3679–3689, 2004.
52. Schmitt-Graff A, Desmouliere A, Gabbiani G. Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. Virchows Arch 425:3–24, 1994.
53. Kedar I, Bleiberg I, Sohar E. Stimulation of murine amyloidosis by a dializable product from pretreated donors. Br J Exp Pathol 56:244–246, 1975.
54. Brandwein SR, Sipe JD, Skinner M, Cohen AS. Effect of colchicine on experimental amyloidosis in two CBA/J mouse models. Chronic inflammatory stimulation and administration of amyloid-enhancing factor during acute inflammation. Lab Invest 52:319–325, 1985.
55. Shtrasburg S, Pras M, Gal R, Salai M, Livneh A. Inhibition of the second phase of amyloidogenesis in a mouse model by a single-dose colchicine regimen. J Lab Clin Med 138:107–111, 2001.
56. Shirahama T, Cohen AS. Blockage of amyloid induction by colchicine in an animal model. J Exp Med 140:1102–1107, 1974.
57. Kisilevsky R, Axelrad M, Corbett W, Brunet S, Scott F. The role of inflammatory cells in the pathogenesis of amyloidosis. Lab Invest 37: 544–553, 1977.
58. Bustorff MM, Oliveria JP, Moura C, Carvalho E, Faria V, Guerra L. Muckle-Wells syndrome nephropathy: lack of response to colchicine therapy. Nephrol Dial Transplant 10:709–710, 1995.