The Evaluation of the Role of *Mycobacterium tuberculosis* in the Etiopathogenesis of Familial Mediterranean Fever

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SUMMARY

Tuberculosis (TB) and familial Mediterranean fever (FMF) are two common diseases in our region, Turkey. Both share some properties in common such that both cause AA type amyloidosis and have association with some immunological abnormalities. Upon incidentally observing M. tuberculosis in bone marrow biopsies of three patients with FMF in a previous study, we intended to elucidate this association prospectively. In this preliminary study, we examined 10 FMF patients, 5 male and 5 female, with a median duration of 17 years disease activity. All were under colchicine therapy. They had no sign of renal involvement. The bone marrow biopsies of these patients were examined for the presence of M. tuberculosis by polymerase chain reaction, BACTEC culture and pathological stains. Pathological examination was performed for the existence of granuloma and amyloid deposition by hematoxilen-eosine, crystal violet and Congo red stains. Negative results were obtained in all specimens examined with all these mentioned methods. The patients had a positive family history of 60% for tuberculosis and in 80% of them with positive tuberculin skin test. We concluded that although there seemed to be a kind of association between both diseases, this relationship is not via the direct existence of bacteria itself. Considering high family history and skin test positivity, one should look for the presence of autoimmune mechanisms under this suspicious relationship between tuberculosis and FMF. Additionally, this became the first study examined the state of amyloidosis in the bone marrow at an earlier stage of FMF without overt renal findings.

Key Words: *Familial Mediterranean fever, tuberculosis, bone marrow, polymerase chain reaction*

ÖZET

Mycobacterium tuberculosis'in Ailevi Akdeniz Ateşi Etyopatogenezindeki Rolünün Araştırılması

Tüberküloz (TB) ve ailevi akdeniz Ateşi (FMF), bölgemizde sık olarak rastlanılan ve bazı ortak özelliklere sahip iki hastalıktır. Her iki hastalıkta da AA tip amiloid depolanması olmakta, her ikisine de bazı immünolojik bozukluklar eşlik edebilmektedir. Daha önceki bir çalışmada, polimeraz zincir reaksiyonu (PCR) metodu ile FMF'li üç hastanın kemik iliğinde tüberküloz basili saptanması üzerine, bu birlikteliği aydınlatmak üzere prospektif bir çalışma planladık. Bu ön çalışmada, ortalama hastalık süreleri 17 yıl olan, 10 FMF hastası (5 erkek, 5 kadın) yer aldı. Hepsi kolşisin tedavisi altında idi. Renal tutuluma ait bulguları yoktu. Alınan kemik iliği as-

pirasyonları ve biyopsileri, M. tuberculosis varlığı açısından PCR ile, kültürü yapılarak ve patolojik olarak incelendi. Patolojik incelemede hematoksilen-eozin, Kongo kırmızısı ve kristal violet boyaları kullanıldı. On hastanın tümünde yukarıda tüm metodlarla tüberküloz basili varlığı gösterilemedi. Hastalarda tüberküloz için aile öyküsü %60 hastada pozitif iken, ppd pozitifliği %80 vakada görüldü. Her iki hastalık arasında bazı ortak bulgular, ilginç bir birlikteliğe işaret etse de, bu birliktelikte direkt olarak mikroorganizmanın varlığı etken değildir. Vakalarımız da yüksek aile öyküsü ve ppd pozitifliği düşünülecek olursa, otoimmün bazı diğer mekanizmaların da araştırılması fikrini doğurmaktadır. Ek olarak, bu araştırma, uzun süreli, bariz böbrek tutulumu olmayan FMF hastalarında, kemik iliğinde amiloid yokluğunu gösteren bizce bilinen ilk sunumdur.

Anahtar Kelimeler: *Ailevi Akdeniz ateşi, tüberküloz, kemik iliği, polimeraz zincir reaksiyonu*

INTRODUCTION

Familial Mediterranean fever (FMF) is a hereditary disease, which almost exclusively affects people of Mediterranean or Jewish, Turks and Arabs. It is characterized by paroxysmal attacks of fever, peritonitis and/or pleuritis often occurring in association with migratory nondeforming arthritis. The diagnosis of FMF is based on clinical evaluation of the patient (1,2). Although the genetic/biochemical basis of the disease is unknown, some events that occur during the period of acute attack are under investigation as possible causes or steps in the etiopathogenesis. Among these are exhaustion of tumor necrosis factor (3), enhanced dopamine beta hydroxylase activity (4) and elevated serum free fatty acids (5) which are injurious to cell membranes and may contribute to the polyserositis which occurs during an attack. Inborn errors of catecholamine metabolism (6), circulating immune complexes (7), leukocyte chemotaxis (8) and complement system abnormalities (9) have all been investigated as potential etiologies for FMF. There are reports of an elevated T4/T8 ratio in FMF, which is corrected by colchicine therapy (10). This might represent a hypernormal T helper cell response to an unknown antigen. We have reported recently that *M. tuberculosis* might be one of the possible triggering factors in the initialization of clinical picture of FMF (11). Both FMF and tuberculosis (TB) are common in our region (12,13). This association should be elucidated. That is why we designed a prospective study. The Study was also first in that it lightened the situation of amyloidosis in the bone marrow of FMF patients without overt renal involvement.

PATIENTS and METHODS

Patient population: There were 5 male and 5 female patients involved in the study with a median age of 31 years old (range 19-47). The median duration of disease was 17 years with a range of 7-39 years. All patients were taking colchicine 1-2 mg daily at the time of study and free of attacks for at least three months. The patients were questioned about family history of FMF and TB, age of onset, the duration of illness, the duration passed without treatment before diagnosis. The fibrinogen level and urinary protein excretion were measured. Tuberculin skin test was performed to all patients. The bone marrow biopsies obtained were examined pathologically for the presence of granuloma and amyloid accumulation. The fresh bone marrow aspiration material was cultured via BACTEC system, and also analyzed by polymerase chain reaction (PCR) for the presence of *M. tuberculosis* genome. We performed tuberculin skin test intradermally with 5U ppd solution in the volar surface of the forehand. The diameter of induration more than 10 mm was considered as a positive test.

PCR: *Sample preparation*: Sections of formalin-fixed, paraffin embedded tissues were scraped with a sterile surgical blade (a new one for each sample) and the scrapings were collected in a 1.5 ml eppendorf tube. After deparaffinization with xylene and ethanol, the samples were incubated at 55°C in a buffer containing 10mM Tris pH 8.0, 0.5% SDS, 100mM NaCl, 25 mM EDTA and, 0.5 mg/ml proteinase-K for 24 hours followed by phenol-chloroform extraction and ethanol precipitation. The resultant dried pellets were suspended in 30 ml distilled water. Five μl from the aqueous suspension was used for PCR amplification. A crude lysate of 1000 *M. tuberculosis* bacteria obtained from a clinical isolate was prepared by boiling 10 min in 1 ml TE solution (10 mM Tris-HCL pH 8.0, 1mM EDTA). This material was used as the positive control for each PCR test.

Amplification: Identical amplification procedures were used with both primer sets. The sample amplified consisted of a 50 μl volume containing 50 mM KCL, 10 mM Tris-HCI pH 9.0, 1% Triton X-100, 2.0 mM MgCl2, 50 mM of each dNTP, 20 pmol of each primer, 1 unit of taq polymerase (Promega). Forty cycles of 94°C 30 sec, 55°C 1 min and 72°C 1 min were performed in an automated thermalcycler. Re-

action mixtures without DNA were used as negative controls. After adjusting the turbidity of the clinical isolate of *M. tuberculosis* to a 0.5 McFarland standard, 10-fold dilutions of the resultant solution were cultured on Lowenstein-Jensen medium. The *M. tuberculosis* detection limit for the PCR procedure was determined by PCR amplification of crude cell lysates prepared from these samples.

Detection of amplification product: The presence of the 123 and 240 bp amplification products was determined by electrophoresis of a 10 ml aliquot of the amplified mixture on a 2% agarose gel. The gels were stained by ethidium bromide and photographed using an UV-transilluminator. Detection of an amplified band with either one of the primer sets was considered as a positive result. The PCR results for each sample were repeated after changing the sample code numbers and only consistently positive samples were reported as positive.

BACTEC culture: Five ml bone marrow aspiration material was incubated into a culture media named 13A specific to mycobacterium. 0.5 cc Bactec supplement was added to the whole mixture. The presence of positive culture was controlled via Bactec 460 instrument for a maximum of 50 days.

Histopathological examination: All bone marrow biopsies were fixed in buffered formalin solution. After decalcification and tissue processing, sections in 5 μm of thickness were obtained from paraffin-embedded tissue. Each section was stained with hematoxylin-eosin, crystal violet and Congo red.

RESULTS

Table 1 demonstrated the summary of the results. The median fibrinogen levels during silent period were 312 mg/dcl (range 200-628 mg/dcl). All but 2 patients had a ppd reaction with a diameter over 10 mm. The median duration of period passed without taking a therapy was 8.5 years with a range of 1-32 years. These patients were suffering a median number of 24 attacks yearly before the administration of colchicine treatment. The median age of onset of FMF in these patients was 10 years with a range of 1-38 years. Seven of 10 patients had a family history of FMF. Four of them had more than one FMF patient within the family. Six of 10 patients had also positive family history for tuberculosis whom had been given antituberculosis treatment. All patients were undergone BCG vaccination in infancy. None of the patient had protein over 75 mg/dcl in urine analysis,

Table 1. *The overall results of the study with respect to examined parameters.*

Number of patients	10
Gender	
Male	5
Female	5
Median age of onset (years)	10
Median duration of illness (years)	17 (7-39)
Median duration passed without treatment before diagnosis (years)	8.5 (1-32)
Median attacks yearly before treatment	24
Positive family history for FMF	70%
Positive family history for tuberculosis	60%
Urinalysis	No abnormal proteinuria
BCG vaccimation	positive in all
Tuberculin skin reaction	over 10 mm in 80%
Median fibrinogen plasma level in silent period	312 mg/dcl
Granulome in bone marrow	negative in all
Amiloid deposition in bone marrow	negative in all
PCR examination of bone marrow for tuberculosis	negative in all
BACTEC culture	negative in all

Table 2. *The individual parameters of all patients are presented.*

Patient no	Age	Sex	Age of onset	PPD	BCG	Duration of illness	Duration without treatment	Urinary protein mg/dcl	Attacks yearly before treatment	Family history for FMF	Family history for TB	Granuloma / amyloid	Culture	PCR
1	31	M	23	20	+	8	1	-	12	-	-	-/-	-	-
2	43	M	6	8	+	37	32	25	24	+	+	-/-	-	-
3	45	F	38	12	+	7	2	1	12	+	+	-/-	-	-
4	19	F	4	0	+	15	1	1	99	+	+	-/-	-	-
5	22	F	1	15	+	20	7	1	99	+	+	-/-	-	-
6	47	M	8	10	+	39	12	25	50	+	-	-/-	-	-
7	37	M	18	15	+	19	15	-	24	-	+	-/-	-	-
8	24	F	12	11	+	12	10	75	15	+	+	-/-	-	-
9	31	F	7	12	+	24	16	75	50	+	-	-/-	-	-
10	29	M	22	10	+	7	4	-	12	-	-	-/-	-	-

which was in the upper normal range of the laboratory. The blood urea and creatine levels of all patients were in normal limits. Table 2 shows the results of the patients individually. We could not detect the presence of *M. tuberculosis* in bone marrow aspiration and biopsy specimens by any of the methods described above.

DISCUSSION

There are some properties common in both tuberculosis and FMF. Both are epidemic in our country (2,12,13). Both could result in AA type amyloidosis. Hypersensitivity type autoimmune reaction takes place somewhere in their courses. For example, in case of tuberculosis, tuberculin skin test was based on this phenomenon (14,15). For these reasons and our observation demonstrating *M. tuberculosis* bacilli in the bone marrow biopsies of three patients with FMF (11), in a ratio more than that detected in the bone marrow of healthy controls as in another study (16), we wanted to investigate whether this finding was just a coincidence or *M. tuberculosis* might have been a cause for the development of FMF. As a result of this preliminary study, we did not demonstrate any evidence for the actual presence of *M. tuberculosis* in any of the patient via any of the above methods. On the other hand, the presence of family history in 60% patients and positive tuberculin skin tests in 80% of cases remains some questions in mind. Could the primary infection of tuberculosis later turn somehow to FMF as a kind of autoimmune phenomenon. If it is so then how should we explain the attacks? Could BCG be a cause for this distorted response of autoimmunity leading to development of FMF by modifying activity of defense system against *M. tuberculosis*?

Diagnostic value of bone marrow biopsy for amyloidosis was first studied by Sungur et al (17), in the patients with renal disease secondary to FMF. They proved that bone marrow was a good site to demonstrate the systemic amyloidosis with a 79.9% success. On the other hand, up to now, the diagnostic value of bone marrow biopsy in earlier stages of the disease, when clinical signs of renal amyloidosis was not evident, was not determined in any study. In our study, however we enlightened to some degree the situation of amyloidosis in patients with FMF who were asymptomatic with respect to renal disease. All of our patients were at preclinical stage with respect to renal involvement, because they all had normal blood urea and creatine levels and no abnormal proteinuria. We could state that there was no amyloid deposition in bone marrow of patients with FMF in preclinical stage, even though they had spent a time having the disease for a median duration of 17 years and 8.5 years of this period had passed without taking any treatment.

Amyloidosis of FMF is much more determined genetically and is not associated with the presence, frequency or severity of the febrile attacks (1,18,19). Turkey could be regarded epidemic for the amyloidosis of FMF (20,21). Tınaztepe et al (21), reported a series of childhood renal amyloidosis secondary to FMF (n=34, 60% of cases with amyloidosis). The median age of the patients was around fourteen. But their

condition of taking colchicine treatment was not clear. With these epidemiological data of Turkey, we would expect to see the development of amyloidosis in our 10 patients. But we also know that the usage of colchicine is very beneficial in prevention, arrest and regression of amyloidosis of FMF. Zemer et al, demonstrated that colchicine treatment of 6-13 years prevented the development of amyloidosis in 350 children, all younger than 16-year-old. All the patients in our study were on the colchicine treatment.

As a conclusion, 1) we did not demonstrate the existence of *M. tuberculosis* in bone marrow of ten patients with FMF. But the high rate of positive tuberculin skin reaction and family history for tuberculosis remains to be elucidated. 2) Colchicine treatment was quite effective in preventing amyloidosis in Turkish patients as well (even though sample size is small). 3) Bone marrow examination yielded no amyloid deposition in preclinical stage of renal involvement in patients with FMF even after a median duration of 31 years of disease.

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