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Absence of mycobacterial DNA in peripheral blood and artery specimens in patients with Takayasu arteritis

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Abstract

The objective of this study was to demonstrate the presence of mycobacterial nucleic acid sequences in peripheral blood and arteries from patients with Takayasu arteritis (TA). Polymerase chain reaction was performed to detect mycobacterial DNA from three different nucleic acid sequences including the insertion sequence (IS) *6110*, the 65-kDa heat shock protein gene (*HSP65*), and the 16S ribosomal RNA (rRNA) gene in peripheral blood from 32 TA patients and in arterial specimens from 10 TA patients. Twenty-eight HIV-negative patients with pulmonary tuberculosis prior to therapy were tested for IS*6110* in peripheral blood as positive controls, and 24 blood donors were evaluated as healthy controls (HC). All TA patients were negative for the insertion sequence IS*6110* and for *HSP65* and 16S rRNA genes in blood samples and in arterial specimens. IS*6110* sequence was found in peripheral blood from 22 (78.5 %) patients with pulmonary tuberculosis but not in HC. In conclusion, the strategy of mycobacterial-specific nucleic acid amplification in the peripheral blood and arterial specimens of TA patients was unable to lend support to the association between TA and tuberculosis long suggested in the literature.

Keywords

DNA; *Mycobacterium tuberculosis*; Polymerase chain reaction; Takayasu arteritis

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Compliance with ethical standards The study protocol was approved by the Institution Ethics Committee and study participants or their relatives gave written informed consent.

Disclosures None.

Introduction

Takayasu arteritis (TA) is a large vessel vasculitis that involves the aorta, its main branches, and the pulmonary arteries [1, 2]. The relationship between TA and tuberculosis has been suggested due to the higher prevalence of TA in countries with a high prevalence of tuberculosis and due to the granulomatous nature of both diseases [3, 4]. Coexisting TA with pulmonary or extra-pulmonary tuberculosis and an increased frequency of positive tuberculin skin test (TST) has been reported in some TA cohort studies [5–7]. Patients with TA have been reported to present an increased humoral and cellular immune response against mycobacterial antigens including the 65-kDa heat-shock protein (HSP65) as well as T cells reactive to mycobacterial HSP65 and to human 60-kDa HSP. These findings suggest a role for *Mycobacterium tuberculosis* in the pathogenesis of TA [8–11]. However, the frequency of latent tuberculosis evaluated by QuantiFERON-TB test is not higher in TA patients in comparison to controls, and the presence of *M. tuberculosis* has never been directly demonstrated either in active or inactive arterial lesions of TA using sensitive techniques in biopsies such as acid-fast- and auramine-fluorochrome-staining methods, mycobacterial cultures, and direct amplification test [12, 13]. The aim of this study was to evaluate whether nucleic acid sequence for the insertion sequence *6110* (IS*6110*), as well as for mycobacterial HSP65 and 16S ribosomal RNA genes could be detected in the peripheral blood and in arterial samples from patients with TA.

Materials and methods

Study population

Fifty-two patients fulfilling the American College of Rheumatology classification criteria for TA were included in the study [14]. Blood samples were collected from 35 patients with TA, 28 HIV-negative patients with pulmonary tuberculosis, and 24 blood donors as healthy controls (HC). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood containing EDTA by density gradient using Ficoll-Hipaque. Paraffin-embedded tissue samples of the arteries were obtained from 17 patients with TA who underwent surgical revascularization procedures or autopsy, and serial 10-µm cuts were performed on tissue samples for DNA extraction. All arterial samples from TA patients were negative for acid-fast bacilli by Ziehl-Neelsen staining. An artery from a patient with known arterial tuberculosis was used as a positive tissue control. The study protocol was approved by the Institution Ethics Committee and study participants or their relatives gave written informed consent.

DNA extraction and PCR analysis

Tissue samples were processed as outlined by the DNeasy Protocol for Animal Tissues [15], using xylene prior to paraffin removal (MERCK, Germany). DNA was extracted from PBMC and arterial specimens using a Qiagen DNAeasy extraction Kit (Qiagen, Valencia, USA). To lyse the mycobacterial wall, 60 µL of proteinase K (20mg/mL) and 50 µL lysozyme (50 mg/mL) were added before DNA extraction. All extracted DNA was stored at –20 °C until polymerase chain reaction (PCR) analysis was performed.

DNA concentration was determined by spectrophotometry at 260 nm, and the purity of nucleic acid extracted was estimated by the ratio of readings at 260 and 280 nm (Genequant/Amersham Biosciences). To ensure that the extracted DNA was appropriate for amplification, the human β -actin gene was amplified (forward primer 5'ATCATGTTTGAGAC CTTCAAC3'; reverse primer 5'CAGGAAGGAAGGCT GGAAGAG3') in DNA from PBMC and another β -actin gene-specific primer set (forward 5'AGCGGGAATCGTG CGTG3' and reverse 5'GGTGATGACCTGGCCGTC3') was used to amplify the same gene in arterial specimens. Only samples that amplified for human β -actin gene were tested for mycobacterial nucleic acid sequences and genes.

The following primers were used to amplify a fragment of 245 bp from *IS6110*: INS1 (5'CGTGAGGGCATCGA GGTGGC3') and INS2 (5'GCGTAGGCGTCGGTGACAAA3') and *M. smegmatis* 1008 strain, which contains a recombinant plasmid bearing the *IS6110* sequence [16] was used as a positive control. The primers IS1 (5'CCTGCGAGCGTAGGCGTCGG3') and IS2 (5'CTCGTCCAGCGCCGCTTCGG3') were used to amplify a 123-bp amplicon in the *IS6110* sequence in arterial specimens. To amplify the *HSP65* gene, the following primers were used: forward (5'GCCAAGAACACCGATGACGT3') and reverse (5'GGTGATGACGCCCTCGTTGC3').

The technique of nested PCR used to amplify mycobacterial 16S rRNA gene involved a first step with the following primers: FO16S (5'GATAAGCCTGGGAACTGGGTC3') and RO16S (5'TTCTCCACCTACCGTCAATCCG3') to amplify a 344-bp fragment, and a second step with primers FI16S (5'CATGTCTTGTGGTGGAAAGCG3') and RO16S (5'TACCGTCAATCCGAGAGAACCC3') yielding a 288-bp fragment of the 16S rRNA gene. All PCR reactions were processed in a 480 thermocycler (Perkin-Elmer, Wellesley, MA). The PCR products underwent electrophoresis on 2 % agarose gels in 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (TAE) buffer and were stained with ethidium bromide to be visualized under UV light. Images were obtained by KODAK system.

Results

TA patients had a mean age of 35.2 years (range 13–53). Female gender comprised 85.7 % of TA patients. Only three patients were Japanese descents, and the other ones had the typical Brazilian European-African-blended ascendancy. No patient had evidence of active tuberculosis or a past history of pulmonary or extra-pulmonary tuberculosis. Information about TST was available for 10 patients with TA and an induration above 15 mm was found in six cases. The human β -actin gene was appropriately amplified in 32 (91.4 %) PBMC samples and in 10 (58.8 %) arterial specimens from TA patients. No nucleic acid sequence from *IS6110* or from *HSP65* gene as well as 16S rRNA gene could be found in PBMC from 32 TA patients. Although, the nucleic acid sequence for the *IS6110* was detected in the arterial sample from the one patient with known arterial tuberculosis, it was not found in artery tissue samples obtained from autopsies or from surgical procedures in 10 TA patients (Table 1). Nonetheless, the nucleic acid sequence from *IS6110* was found in PBMC from 22 (78.5 %) patients with active pulmonary tuberculosis (Table 1) but not in peripheral blood from TA patients or HC.

Discussion

In this study, the presence of mycobacterium-specific nucleic acid sequences could not be detected either in PBMC or in paraffin-embedded arterial samples from patients with TA. These findings are in line with the results from Arnoud et al. that could not observe direct mycobacterium infection in arteries from TA patients with active and inactive inflammatory process [13], indicating that the presence of mycobacterium in peripheral blood or in arterial tissue is not required for the maintenance of arterial inflammation in TA. Furthermore, the similar prevalence of latent tuberculosis in TA patients and controls suggests that extra-arterial latent infection would not be responsible for triggering the arterial inflammatory process [12].

The decision to evaluate the *IS6110* sequence and mycobacterial *HSP65* and 16S rRNA genes in this study aimed to cover all mycobacterium species besides *M. tuberculosis* [17]. A potential limitation of this study is the extraction of DNA from paraffin-embedded arteries, since this type of samples usually presents degraded DNA. However, the use of only arterial specimens that were positive for human β -actin gene minimized this potential pitfall. In addition, the positive amplification of mycobacterium *IS6110* nucleic acid sequence in a specimen from a patient with tuberculosis arteritis served as a positive control for such type of material.

Several studies show pieces of evidence suggesting the involvement of *M. tuberculosis* in the pathogenesis of TA. A higher frequency of TST intradermal reaction to specific antigens to other mycobacterium species such as *M. kansasii* and *M. avium* has been described in TA patients in comparison to control subjects [18]. The prevalence of IgG, IgM, and IgA antibodies against mycobacterial HSP65 is higher in TA patients than in controls. The same is true for antibodies against 60-kDa human HSP [9, 11]. The strong correlation between antibodies against mycobacterial HSP65 and against human HSP60 ($r = 0.814$; $p < 0.001$) indicates that the loss of tolerance to stress induced molecules in TA might be in part due to a molecular mimicry mechanism triggered by a previous mycobacterial infection [9–11, 18]. Indeed, γ/δ T cells from patients with TA exhibit reactivity to human HSP60 and cytotoxicity to aortic endothelial cells indicating that response against human HSP60 may be one of the inciting events in the pathogenesis of TA [19].

Recently, several case series have reported good results with the use of TNF α antagonists, including infliximab, etanercept, and adalimumab, in TA patients refractory to conventional immunosuppressive therapy. Although the link between TA and tuberculosis is still an open question, the use of anti-TNF α agents in TA patients has not been associated with an increased rate of mycobacterial infection [20]. Of interest, none of the TA patients evaluated in the present study had ever been treated with anti-TNF α agents.

In conclusion, despite the presence of indirect evidence of association between TA and mycobacterial infection, we could not find mycobacterial DNA in PBMC or in tissue samples from the arteries of patients with established TA. This indicates that mycobacterial infection may be a triggering event for disease development but sustained mycobacterial infection is not necessary for maintenance of arterial inflammation in TA.

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Table 1

IS6110 nucleic acid sequence in arteries and in peripheral blood of patients with Takayasu arteritis and in patients with tuberculosis

Groups	Presence of IS6110 nucleic acid sequence in PBMC (%)	Presence of IS6110 nucleic acid sequence in arterial specimens (%)
Takayasu arteritis	0 (0.0)	0 (0.0)
Pulmonary tuberculosis	22 (78.5)	NA
Arterial tuberculosis	NA	1 (100.0)

The number of patients with Takayasu arteritis and pulmonary tuberculosis who underwent PCR for IS6110 nucleic acid sequence of in PBMC was 32 and 28, respectively, whereas 10 arterial specimens from patients with Takayasu arteritis were analyzed for IS6110 nucleic acid sequence

NA not applicable, PBMC peripheral blood mononuclear cells