Published in final edited form as:

Am J Med Sci. 2009 April ; 337(4): 233–235.

Lack of evidence for bacterial infections in skin in patients with systemic sclerosis

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Abstract

Background—In some patients with systemic sclerosis (SSc), persistent bacterial infection involving dermal microvascular endothelial cells may result in endothelial injury, leading to the obliterative micro-vasculopathy typical of the disease. Alternatively, in some SSc patients persistent bacterial infection involving activated dermal fibroblasts or other cells found in scleroderma skin might result in the fibrosing features of this disease. In this study, we investigated bacterial infection in skin in SSc patients.

Methods—Chlamydiae of many species are known to undergo persistent infection. Highly sensitive and specific PCR assays targeting chromosomal DNA sequences from *C. trachomatis* and *C. pneumoniae* were employed to screen skin biopsy samples from each of 18 patient and 26 control individuals. Additional screening was done using a highly sensitive “pan-bacteria” PCR screening system.

Results—All patient and control samples proved to be PCR-negative for both chlamydial species. Similarly, all patient and control samples were PCR-negative when the broad range “pan-bacteria” assay system was employed.

Conclusion—While some caveats apply, the data presented here do not support the contention that persistent bacterial infections play an important role in the pathogenesis of SSc.

Keywords

Systemic sclerosis; persistent bacterial infection; *C. trachomatis*; *C. pneumoniae*; PCR-based screening

Introduction

Systemic sclerosis (SSc) is a chronic disease with characteristic microvasculopathy and a typical pattern of dermal and internal organ fibrosis^{1,2}. The small vessel obliterative vasculopathy is marked by intimal thickening, luminal narrowing, enhanced vasospasm, and a reduced capillary bed. The clinical consequences of these vascular changes include Raynaud’s phenomenon, critical digital ischemia, pulmonary arterial hypertension, and

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episodic hyper-reninemic hypertension (scleroderma renal crisis)². Along with vascular abnormality, fibroblast activation is common, leading to enhanced collagen production and pathologic accumulation resulting in fibrosis of the skin¹. Coincidental with, or subsequent to, dermal fibrosis is the development of internal organ fibrosis. This is accompanied in SSc by the presence of autoantibodies (auto-Ab), with ~90% of patients having antinuclear Ab (ANA) similar to that seen in systemic lupus erythematosus (SLE)³. The connection between the vascular abnormalities and the fibrosing features in scleroderma is not well understood.

Scleroderma small vessel vasculopathy shares some key features with large vessel atherosclerosis, a condition also characterized by intimal proliferation and luminal narrowing, among other abnormalities⁴. Inflammation plays an important role in the pathogenesis of atherosclerosis, raising the possibility of infectious agents as mediators in this process^{5,6}. A link between typical macrovascular atherosclerotic disease and scleroderma has been reported but remains tenuous⁷. However, particular factors, either host or infectious agent-specific, may predispose scleroderma patients to small vessel involvement.

No cure exists for scleroderma, and therapy aimed at ameliorating symptoms usually shows only limited success⁸. Lifespan is shortened, and in the most severe cases markedly shortened, in patients with the disease. The cause is unknown but is generally thought to be a combination of factors requiring an external trigger or triggers acting on a genetically predisposed host. Given this incomplete state of knowledge, demonstration of a persistent bacterial infection in key target sites in at least a subset of patients may provide useful information regarding likely pathogenic mechanisms and a potentially useful treatment approach. The objective of this study was to assess the possibility that in some patients with SSc, persistent bacterial infection involving dermal microvascular endothelial cells results in endothelial injury, leading to the obliterative microvasculopathy typical of the disease. Alternatively, in some SSc patients a persistent bacterial infection involving activated dermal fibroblasts or other cells found in scleroderma skin may result in the fibrosing features of this disease. For this study, we targeted the human bacterial pathogens *Chlamydia trachomatis* and *C. pneumoniae* since they are highly prevalent in most populations studied and are known to undergo persistent infection in humans⁹ for review. We also employed a broad range assay system to assess the presence of other bacterial species in the samples examined.

Materials and methods

Patients

All patients met the 1980 American Rheumatism Association (now the American College of Rheumatology) classification criteria for Systemic Sclerosis and were selected on the basis of willingness to participate in the study (Table 1)¹⁰. The distinction of limited vs. diffuse cutaneous disease was made according to the system of LeRoy *et al.*¹¹; for purposes of this study, limited disease was considered to include the skin of the forearm, rather than only the fingers or back of the hand. Disease duration was based on the time between the first non-Raynaud's phenomenon symptom characteristic of SSc and the date of biopsy. Samples from healthy control individuals were obtained from friends of cases and medical center staff. A questionnaire was administered to potential control subjects regarding signs and symptoms of Raynaud's phenomenon, as well as personal history of, or first-degree family member with, a diagnosed autoimmune connective tissue disease. Only those with negative responses were included as controls. This study was approved by the Human Subjects Committees of Wayne State University and the University of Texas – Houston Medical Schools.

Autoantibody Determinations

ANA testing was done by indirect immunofluorescence (IIF) using HEp-2 cells as antigen substrate (Inova Diagnostics, San Diego, CA). Sera were screened at 1/80 dilution. Anti-centromere Ab were defined by their characteristic IIF patterns. Sera were further tested for anti-topoisomerase Ab by passive immunodiffusion against calf thymus extracts, using a commercial kit (Inova Diagnostics). Ab to RNA-polymerase III were tested using an enzyme-linked immunosorbent assay (ELISA) kit from Medical & Biological Laboratories Co., LTD. (Nagoya, Japan).

Skin Biopsies

Skin biopsies were done using a 4 mm punch instrument under local anesthesia, following surface sterilization of the skin with iodine then an alcohol swab. The samples were taken from the same site on all subjects - the dorsal surface of the forearm at 2–3 inches proximal to the wrist crease, and in all cases of patients with SSc biopsies were obtained from involved skin. Peripheral blood was drawn at the time of skin biopsy. Two subjects (one case and one control) provided skin biopsies but not blood samples due to technical difficulties. Biopsies were immediately frozen at -80°C and sent to the laboratory for PCR screening analyses (see below). Blood samples were not frozen and were sent to the laboratory at the same time as were biopsy samples.

Preparation and analysis of DNA

Total DNA was prepared from skin biopsies by the hot phenol method, as described by us^{6,8, 12–15}. Preparation of DNA from blood samples was done using a kit (Flexigene®, Qiagen Inc., Valencia, CA). PCR assays targeting chromosomal DNA sequences from *C. trachomatis* and *C. pneumoniae* have been described by us, as has the “pan-bacteria” PCR assay used here; assay conditions and primer sequences used are given in those publications^{12–15}. While the “pan-bacteria” assay does not find all bacterial species, it does identify an extremely wide variety of organisms, including *Staphylococcus aureus*, *Borrelia burgdorferi*, *Salmonella enterica*, *Chlamydia trachomatis*, *C. pneumoniae*, and many others.

Results

Table 1 briefly summarizes demographic features of the subject population. The controls all were female, whereas the case subjects were 61% female. Otherwise there was no significant difference between patients and controls in race/ethnicity or mean age. Mean disease duration among the patients was 46 mo, with a range of 7–74 mo. No significant difference existed in disease duration between those with limited and those with diffuse cutaneous SSc (Table 2). ANA were present in 94% (17/18) of the patients. The single patient with a negative ANA had diffuse cutaneous disease. Scleroderma-specific auto-Ab (anticentromere, antitopoisomerase, or anti-RNA polymerase) were present in 12 patients (66%), as expected³.

PCR screening assays for chromosomal DNA sequences from *C. trachomatis* were uniformly negative for all patient subject and control samples, as were all congruent PCR screening assays targeting *C. pneumoniae* chromosomal DNA sequences (not shown). Extensive experience has demonstrated that it is extremely unlikely that chlamydiae will be identified in blood samples by PCR if they are not identified using these assays in relevant tissue samples. Thus, blood samples were not assayed for either chlamydial species in this study. PCR assays on DNA from the skin biopsies utilizing the “pan-bacteria” primer set were similarly negative for all patient and control samples. However, DNA from each blood sample was assayed using the “pan-bacteria” screening system, and all were negative in those analyses.

Discussion

In this study, we examined skin biopsy samples from individuals with SSc and from normal control individuals to assess the possibility that bacterial infection contributes to pathogenesis in some patients with the disease. We were particularly interested in infections by *C. trachomatis* and/or *C. pneumoniae*, since these organisms are of wide distribution and have been demonstrated to undergo chronic, persistent infection under many circumstances; in some contexts, persistent chlamydial infections are thought, and in some cases known, to engender chronic diseases^{e.g., 9}. We also utilized a “pan-bacteria” primer set in our PCR-based screening assays, and this system has been shown to identify bacteria from a wide variety of prokaryotic genera^{e.g., 15}. The primers for this system target highly conserved regions of the bacterial 16S rRNA gene but have more than 500 bp of less well conserved sequence between them, which is enough under most circumstances to define an unknown organism to the species level.

All PCR screening assays targeting *C. trachomatis* and *C. pneumoniae* in this study proved to be negative. Thus, these universally negative results indicate that infection with either of these two pathogens cannot be the explanation for skin pathology in a majority of SSc cases. However, a role in pathogenesis in some minority of cases remains a possibility, given the relatively limited patient cohort studied here. Because the biopsies assayed were obtained only from involved skin in patients with SSc, a question may remain as to whether dermal fibrosis, extensive or otherwise, might have generated false negative assay results. This seems unlikely to us, since although dermal blood vessels are decreased in SSc many remain, and skin tissue of the forearms remains fairly viable in the disease.

Interestingly, and rather surprisingly to us, all assays that employed the “pan-bacteria” PCR screening system also were negative in all subject samples. While this system has proved to be extremely sensitive and widely general in its ability to “find” bacterial 16S rRNA sequences in our hands, it does not, of course, identify such sequences from all bacterial species. Thus, it is possible that this more generally targeted screening system simply missed organisms present in the patient samples examined. More study will be required to assess this possibility. At this point, however, our data do not support the contention that persistent bacterial infections play an important role in the pathogenesis of SSc. Further, the assay systems used in the present study do not identify viral or other non-bacterial organisms. Several viral pathogens, including parvovirus, cytomegalovirus, and others have been implicated in disease genesis, these organisms thus will require further study in patients with SSc^{e.g., 4,16,17}.

Acknowledgments

Support: This study was supported by grants (R21) AR-48056 (M.D.M.), (RO1) AR-42541 (A.P.H.), and (RO1) AR-48331 (J.A.W.-H.) from the US National Institutes of Health.

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TABLE 1

Demographic Features of the Study Population

FEATURE	CASES (n=18)	CONTROLS (n=26)	p value
Female Sex (%)	11 (61.1%)	26 (100%)	p=0.0001
Race/Ethnicity			
Caucasian	14 * (77.8%)	13 ** (50.0%)	p=0.122
African-American	3 (16.7%)	13 (50.0%)	p=0.052
Asian	1 (5.5%)	0 ----	---
Mean Age $\pm$ sd (range)	47.3 $\pm$ 9.5 (36–65)	46.0 $\pm$ 10.1 (32–61)	p=0.669
Mean Disease Duration in months $\pm$ sd (range)	45.9 $\pm$ 21.2 (7–74)	NA	NA

*
2 cases were Hispanic/Caucasian

**
3 controls were Hispanic/Caucasian

TABLE 2

Disease Characteristics of the Case Population

FEATURE	Cases (n=18)	Disease Duration in months ± sd (range)	p Value
Type of SSc:			
Diffuse cutaneous disease (%)	14 (77.8%)	47.4 ± 21.4 (7–74)	p=0.540
Limited cutaneous disease (%)	4 (22.2%)	40.7 ± 22.9 (14–64)	
ANA positive			
Anticentromere positive	3		
Antitopoisomerase positive	3		
AntiRNA polymerase positive	6		