Physicochemical characterization of inorganic deposits associated with granulomas in cutaneous sarcoidosis

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Running head: Inorganic deposits in skin sarcoidosis

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Abstract

Background. Sarcoidosis, characterized by epithelioid granulomas, is considered to be caused by a complex interplay between genetics and environmental agents. It has been hypothesized that exogenous inorganic particles as crystalline silica could be a causal or adjuvant agent in sarcoidosis onset.

Objectives. To investigate the location, frequency and physicochemical characteristics of foreign materials and mineral tissue deposits in the granulomatous area of cutaneous sarcoidosis.

Methods. Skin biopsies (n=14) from patients diagnosed with cutaneous sarcoidosis (mean age 43 years; 11 patients with extra-cutaneous involvement) were investigated using polarized light examination (PLE), µFourier Transform Infra-Red (µFT-IR) spectroscopy and Field Emission Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy (FE-SEM/EDX)

Results. Combined PLE, µFT-IR, FE-SEM/EDX analysis allowed to characterize mineral deposits in 7/14 biopsies (50%). It identified crystalline silica (SiO_2) inside granulomas in 3 biopsies and calcite ($CaCO_3$) at their periphery in 4.

Conclusion. This study emphasizes the need of using combined methods for assessment of mineral deposits in granulomatous diseases. According to the location and characteristics of deposits we can hypothesize that SiO_2 particles contribute to the granuloma formation, whereas $CaCO_3$ deposits are related to the granuloma biology. However, the significance of the association between SiO_2 deposits and sarcoidosis is still disputed.

Introduction

Sarcoidosis is characterized by an exaggerated granulomatous response mostly affecting the lung, skin and eye, locations particularly exposed to exogenous particulates.[1-2] Granuloma mostly constituted of macrophage lineage cells is thought to be controlled by innate as well as adaptive immune mechanisms in a complex combination of genetic susceptibility, immune networks and infectious and/or environmental agents.[3]

Sarcoidosis affects the skin in 25-30% of patients and manifests by a wide range of clinical cutaneous lesions.[4-5] It is well known that sarcoidosis preferentially affects sites with a prior injury as tattoos or scars. Polarizable material has been reported up to 22% of the cases suggesting that foreign material could be a nidus for granuloma formation and a potential trigger for the disease.[6-8]

This study, based on a series of cutaneous biopsies of sarcoidosis, was designed to investigate the frequency and physicochemical characterization of mineral deposits in the granulomatous areas. To describe their characteristics at the subcellular scale as well as their chemical nature, in addition to polarized light examination (PLE), two different techniques of physicochemical analysis were used: µFourier Transform Infra-Red (µFT-IR) spectroscopy and Field Emission Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy (FE-SEM/EDX).[9] Such a combination of methods has been amply demonstrated to be particularly effective for detection and physicochemical identification of inorganic/ mineral deposits in various tissue samples.[10]

Material and methods

Skin biopsies, histopathology and polarized light examination

The study was conducted in compliance with Good Clinical Practices and the Declaration of Helsinki, according to French law.

A retrospective monocentric study was conducted, from January 2012 to December 2015. Consecutive patients diagnosed with skin sarcoidosis during this period, based on clinical data and histopathology results, were included. Clinical and biological data, including demographic, occupation, past medical history, extra-cutaneous involvement of the disease, clinical description and location of the skin lesions, calcium and angiotensin converting enzyme serum levels were collected.

Five samples from negative margins of skin carcinoma resections were included and further described as controls.

Multiples sections of 1.5 µm from paraffin embedded skin biopsies were obtained and deposited on two types of slides: glass slides, for hematoxylin-eosin-saffron (HES), and low-e microscope slides (MirrIR, Kevley Technologies, Tienta Sciences, Indianapolis) for FE-SEM/EDX and µFT-IR. The location (superficial and deep dermis, sub-cutis) and number of granulomas observed per biopsy were evaluated. A screening by PLE was done by two different pathologists.

Skin sections were analysed and compared between cases and controls, for the presence of abnormal deposits.

Physicochemical analysis of mineral deposits

The tools and methods have been extensively described in a thematic issue of the Comptes Rendus Chimie de l'Académie des Sciences Paris France.[11]

Briefly, for µFT-IR, tissue sections deposited on low-e microscope slides were analyzed with the Spotlight 400 FTIR imaging System (Perkin Elmer Life Sciences - Courtaboeuf, France), in the mid infrared (4000-700 cm-1) spectral range to obtain infrared maps of tissue slides at high spatial resolution, down to 10 µm.[9]

For FE-SEM/EDX the sections previously used for µFT-IR were observed on a Zeiss SUPRA55-VP SEM (Zeiss SUPRA55-VP - Oberkochen, Germany) equipped with in-lens SE and Everharte Thornley SE secondary electron detectors.[12] Energy Dispersive X-ray spectroscopy (EDX) coupled to FE-SEM observation was used to confirm the nature of the deposits.

Results

Skin biopsies, histopathology and polarized light examination (PLE)

Sixteen patients were diagnosed with skin sarcoidosis at Tenon Hospital from January 2012 to December 2015. Two patients were excluded because the tissue sample available for analysis was too small. Finally, cutaneous biopsies from 14 patients (9 men; 5 women) were selected for further analysis. Demographic, clinical and histopathological characteristics of all patients are summarized in Table 1 and for each patient in table 2 (supplementary material). Eleven patients had associated extracutaneous localizations of sarcoidosis mostly pulmonary

(n= 10). One of the three patients without extracutaneous localization, (patient 8 in table 2 supplementary material) had a single granulomatous lesion of the forehead and the diagnosis of "localized sarcoidosis-like granulomatous reaction" could not be excluded. For PLE from 4 to 33 (mean= 16) sections per biopsy were analyzed. Refractive material was detected in 5 biopsies, restricted to one or two granulomas per section (Fig. 1). Interstitial peri granulomatous fibrosis was observed in 13 cases without any polarized signal observed in this area.

Physicochemical analysis of mineral deposits

Intra granuloma mineral silica (SiO_2) deposits

In 3 biopsies with PLE refractive material, µFT-IR characterized mineral silica deposits (Fig. 2).

FE-SEM observation showed that silica particles were inside the granulomas. In two samples, the deposits were sharp, unique, irregular objects more than 10µm long. In the third, the deposit was an aggregate of very small objects some of them at the nanoparticle scale. No other nanoparticles of SiO_2 were observed within the granuloma or in its vicinity. EDX analysis confirmed the presence of silica in these 3 samples (Fig. 2).

Peripheral calcium carbonate ($CaCO_3$) deposits

µFT-IR analysis of 4 biopsies detected calcite ($CaCO_3$) deposits in the form of calcite at the periphery of the granulomas. FE-SEM/EDX showed aggregates of submicrometer spherical

deposits of calcite located between the collagen fibers (Fig. 3). Two of these 4 patients had hypercalcemia.

Controls

Similar analyses were performed on the 5 samples from the control group, no deposits were found either in dermis or subcutis.

4. Discussion

Using combined methods, we yield comprehensive information on the location and physicochemical nature of inorganic deposits in granulomas (crystalline silica SiO_2) and perigranuloma tissues (calcite $CaCO_3$) in patients with cutaneous sarcoidosis. We identified crystalline silica (SiO_2) inside granulomas in 3 biopsies and calcium carbonate ($CaCO_3$) at their periphery in 4.

Refractive material was observed by PLE in 5 biopsies (35%) which is in accordance with the 20%-78% reported range.[7] In our series, µFT-IR showed silica deposits in 3 biopsies (21%) which has been confirmed to be crystalline silica by FE-SEM/EDX. For one of these biopsies the PLE examination was negative, while for 3 PLE positive samples infrared spectroscopy identified calcium carbonate deposits.

Crystalline silica was identified in granulomas in 3 biopsies from environment exposed skin areas (elbow, arm and forehead). This finding is in keeping with the origin of SiO_2 deposits in relation to environmental or occupational exposure. For one patient the area has previously been affected by a trauma which could have been responsible for deposition of foreign

bodies. Accordingly to epidemiological studies, crystalline silica has been suggested to play a role in sarcoidosis occurrence particularly after environmental or occupational exposure.[13] However, in the present study identification of SiO_2 particles has been a particularly rare event affecting at least 1 granuloma section among a mean of 75 analyzed per biopsy. But it cannot be excluded that the frequency of deposits was largely underestimated. As each granuloma has a mean diameter of at least 150-200µm,[14] 5µm sections correspond to a very limited fraction of the lesion. In addition, loss of these hard particles deposits may occur during the processing of tissue sample particularly at the sectioning step. SEM examination showed that SiO_2 particles were observed as unique intracellular deposits. It has been hypothesized that nanoparticles of silica undetected by PLE could be a causative agent in sarcoidosis.[15] The significance of the frequent association between foreign material and sarcoidosis is still controversial.[8] Foreign bodies may be initial triggers for sarcoidosis constituting the nidus for granulomas in patients with a particular genetic background having an impaired capacity to handle particulate foreign material.[16-17] Conversely another hypothesis is to consider granuloma in sarcoidosis as a host related inflammatory/ immune reaction non-specific of a special antigen or particulate occurring at sites where potential incidental triggering agents (scars, foreign material, antigens…) are already located. In line with this hypothesis is the observation of granuloma recurrence on tattoos or scars in parallel with exacerbation or relapse of systemic sarcoidosis.[18]

Calcite deposits were observed at distance from granulomas, within the connective tissue of dermis in 4 patients, two with hypercalcemia. Calcium oxalate and phosphate deposits have been reported in sarcoidosis, however, to the best of our knowledge, we report the first cases of calcite deposits.[19] Presence at the nanometer scale of these deposits, including in patients with normal serum calcium level, raises the question that calcium carbonate deposits in

tissues around granulomas could be a common not yet highlighted finding in conjunction with the role of the granuloma in vitamin D and calcium metabolism.[20]

In conclusion such a study needs to be extended to samples from other organs affected by sarcoidosis, mostly lung and mediastinal lymph nodes exposed to environmental and occupational pollutants. Moreover the consequence of calcite deposits in organs where granulomas develop should be investigated.

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Table 1 Demographic, clinical and histopathological data of cases

	N (%)
Patient's characteristics	14
Men / Female	9/5
Age at inclusion (years)	43 (23-68)
Type of lesions	
Nodules	7 (50)
Papules	6 (43)
Plaques	1 (7)
Localisation	
Lower limbs	5 (36)
Face	5 (36)
Upper Limbs	4 (29)
Trunk	2 (14)
Treatment	
Hydroxychloroquine	7 (50)
Systemic corticosteroids	5 (36)
Immunosuppressive therapy	4 (29)
Local corticosteroids	2 (14)
No treatment	1 (7)
Extra cutaneous localisations	11 (79)
Pulmonary	10 (71)
Renal	2 (14)
Osteo-articular	2 (14)
Neurological	1 (7)
Ophthalmological	1 (7)
Occupational exposure to mineral dusts	4 (29)

Biology	
Hypercalcemia *	2 (14)
Elevated angiotensin converting enzyme level	4 (29)
Number of granulomas per section	
≤ 10	5 (33)
10 to 50	6 (40)
> 50	4 (26)
Localisation of the granulomas	
Superficial dermis	11 (73)
Deep dermis	10 (67)
Hypodermis	5 (33)
Presence of refractive material	5 (33)

* Defined as serum calcium level > 2.6 mmol/l

Figure legends

Figure 1 Optic microscopy, PLE and SEM of granulomas in cutaneous sarcoidosis. (a) Optic microscopy of a characteristic cutaneous granuloma (HES x 200). (b) PLE of the same granuloma, showing birefringent particles. (c) Optical microscopy of another granuloma (HES x 400); (d) PLE, showing birefringent particles. (e-f) SEM photographs showing an intragranuloma inorganic deposit at two magnifications.

Figure 2 Examples of physicochemical analysis of the mineral silica (SiO_2) deposits observed within a granuloma. (a) µFT-IR microspectroscopy: IR spectrum showing the characteristic signals of silica in the protein matrix of a granuloma. (b) FE-SEM/EDX the EDAX spectrum identifies the presence of a significant signal related to silicon (Si) in the deposit (1.740 keV cm^{-1})

Figure 3 Detection and physicochemical analysis of calcite ($CaCO_3$) deposits at the periphery of granulomas. (a) SEM photograph showing aggregates of submicrometer spherical deposits localized between the collagen fibers (arrow). (b) µFT-IR microspectroscopy: IR spectrum showing the characteristic peaks of calcite ($CaCO^3$). (c) FE-SEM/EDX: the EDAX spectrum identifies the presence of a significant signal related to calcium (Ca; 3.7 keV) and the absence of a signal from phosphore (P; 2.01 keV) in line with the FTIR spectrum identifying calcite ($CaCO_3$).

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Neurological	1 (7)
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Deep dermis	10 (67)
Hypodermis	5 (33)
Presence of refractive material	5 (33)

* Defined as serum calcium level > 2.6 mmol/l

Patients			**Clinical presentation**			**Histopathology**			**Calcemia and ACE[3] level**	**PLE[4]**	**Physicochemical characterization of the deposits**
	Gender[1]	age	Skin lesions location	Type of skin lesion	Other organs involved	Location of the biopsy	Number of granulomas[2]	Granulomas location			
1	M	47	Upper and lower limbs	Nodules	None	Lower limb	10	Subcutis	Both normal	Positive	Calcium carbonate
2	M	31	Face	Papules	Lung, joint	Temple	21	Superficial and deep dermis	Hypercal-cemia Elevated ACE	Negative	Calcium carbonate
3	M	55	Lower limbs	Nodules	Lung	Lower limb	76	Deep dermis and subcutis	NA[5]	Positive	Calcium carbonate
4	F	61	Upper limbs	Nodules	Lung	Arm	33	Deep dermis and subcutis	Both normal	Negative	-
5	M	68	Toes	Plaques	Lung	Toe	10	Superficial and deep dermis	NA[5]	Negative	-
6	F	43	Face, upper limbs	Nodules	Lung	Elbow	48	Superficial and deep dermis	Normal calcemia Elevated ACE	Negative	Crystalline silica

7	M	42	Upper limbs	Nodules	Lung	Arm	115	Deep dermis	Both normal	Positive	Crystalline silica
8	F	23	Fore Head	Nodule	NA[5]	Fore Head	70	Superficial and deep dermis	NA[5]	Positive	Crystalline silica
9	M	41	Back	Papules	Kidney	Back	73	Superficial and deep dermis	Hypercal-cemia Normal ACE	Positive	Calcium carbonate
10	F	51	Back	Papules	Lung	Back	7	Superficial dermis	Both normal	Negative	-
11	M	38	Genitals, lower limbs	Nodules	Lung, Central nervous system	Lower limb	33	Deep dermis and subcutis	NA[5]	Negative	-
12	M	45	Lower limbs	Papules	Lung, kidney, eye	Lower limb	22	Superficial dermis, deep dermis and subcutis	Normal calcemia Elevated ACE	Negative	-
13	F	35	Scalp	Papules	None	Scalp	9	Superficial dermis	Both normal	Negative	-
14	M	28	Face	Papules	Lung	Face	19	Superficial and deep dermis	Normal calcemia Elevated ACE	Negative	-

Table 2: Patients, histopathology, biology and deposits characterisation in the 14 skin biopsies for cutaneous sarcoidosis.

[1] M: male; F: female

[2] Number of granulomas screened per biopsy

[3] ACE: angiotensin conversing enzyme

[4] PLE: polarized light examination

[5]NA: not available