Original Full Length Article

Histology-directed and imaging mass spectrometry: An emerging technology in ectopic calcification

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ABSTRACT

The present study was designed to demonstrate the potential of an optimized histology directed protein identification combined with imaging mass spectrometry technology to reveal and identify molecules associated to ectopic calcification in human tissue. As a proof of concept, mineralized and non-mineralized areas were compared within the same dermal tissue obtained from a patient affected by Pseudoxanthoma elasticum, a genetic disorder characterized by calcification only at specific sites of soft connective tissues. Data have been technically validated on a contralateral dermal tissue from the same subject and compared with those from control healthy skin. Results demonstrate that this approach 1) significantly reduces the effects generated by techniques that, disrupting tissue organization, blend data from affected and unaffected areas; 2) demonstrates that, abolishing differences due to inter-individual variability, mineralized and non-mineralized areas within the same sample have a specific protein profile and have a different distribution of molecules; and 3) avoiding the bias of focusing on already known molecules, reveals a number of proteins that have been never related to the disease nor to the calcification process, thus paving the way for the selection of new molecules to be validated as pathogenic or as potential pharmacological targets.

Introduction

Calcium and phosphate deposition in soft connective tissues occurs in a number of genetic diseases, in metabolic disorders, such as uremia, hyperparathyroidism and diabetes, or secondary to inflammation or atherosclerosis. Numerous proteins have been identified to be involved in bone calcification as well as in ectopic mineralization, suggesting that an active and dynamic balance of pro-calcifying and anti-calcifying mechanisms takes place in both physiological and pathological calcifications [1]. Nevertheless it is still unclear whether calcification affects particular matrix components in specific organs/tissues, whereas other areas remain unaffected and which molecules/pathways could be targeted for pharmacological approaches. To address these questions, investigations performed so far have looked at the specific expression/localization of already known proteins [2] or have used cell lines and tissue extracts to pick up unknown gene/proteins by means of "omic" techniques [3–5]. However, the major difficulty of these techniques is the ability to analyze a large number of proteins without losing the morphology and the tissue architecture and, even more importantly, to discriminate which proteins belong to normal or to pathologic areas. Therefore, in this study, on-tissue analyses were carried out by Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) (profiling and imaging) [6,7] as a consolidated tool for the analysis of biological and clinical tissue samples [8–12]. This approach has several advantages: i) endogenous (locally synthesized by cells) as well as exogenous (derived from the blood stream) molecules can be analyzed directly from the tissue in their native environment, without homogenization, thus preserving spatial relationship of molecules within a specimen; ii) it does not require the use of antibodies; and iii) it can map the expression of hundreds of proteins from a single (8 μm thick) tissue section.

However, MALDI MS, although leading to the detection of a large number of peptides and small proteins (up to 25 kDa), cannot be currently utilized for larger proteins (exceeding 25 kDa). In order to detect also proteins larger than 25 kDa, we have applied a histology-directed mass spectrometry protein identification [13] using hydrogel discs as carriers for the enzyme, thus allowing the digestion to take

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place directly on discrete tissue areas preserving the relationship between molecular information and tissue architecture.

As the technology advances, the application of MALDI MS as well as of miniaturized hydrogel devices for histology directed on-tissue protein digestion in the clinic will continue to expand, enabling to play a central role in the diagnosis and prognosis of diseases and in the evaluation of patient's therapy.

Therefore, we have combined this MS-based technology in order to investigate skin biopsies from a patient affected by Pseudoxanthoma elasticum, a rare genetic disorder characterized by a progressive calcification occurring in specific areas of soft connective tissues, whereas other regions remain unaffected [14]. Proteomic analyses were performed on mineralized and non-mineralized areas of the same biopsy, and data compared with those from a control healthy tissue.

Material and methods

Tissue specimen collection

The patient was a woman 46 years old affected by Pseudoxanthoma elasticum (PXE). The disease was clinically diagnosed at the age of 15 years by the presence of typical dermal alterations (Fig. 1A) and by ocular angioid streaks. Biomolecular analyses confirmed the clinical diagnosis of PXE revealing two causative missense mutations in the ABCC6 gene: one on exon 12 (c.1553G>A, p.Arg518Gln) and the other on exon 24 (c.3389C>T, p.Thr1130Met).

Control tissue was obtained from a woman 47 years old undergoing elective cosmetic surgical procedures. No connective tissue alterations were present, nor any clinically relevant condition.

Consent was obtained to use these specimens for research purposes in accordance with the Declaration of Helsinki protocol.

After surgery, skin samples were immediately placed in fixatives for morphological analyses or frozen in liquid nitrogen and stored at −80 °C until ready for processing and preparation for mass spectrometry analysis.

Light and electron microscopy

For the demonstration of calcified elastic fibers, skin specimens (approximately 1 cm^3) were routinely fixed in 10% (v/v) formalin in water, dehydrated and embedded in paraffin. Five to seven micron thick sections were collected on glass slides and processed for the von Kossa stain. Briefly, sections were deparaffinized and hydrated, stained for approximately 1 h with 5% (w/v) silver nitrate in water under a UV-lamp, rinsed with water, immersed for 5 min in 5% (w/v) thiosulfate in water and finally observed with a Zeiss Axiophot light Microscope (Jena, Germany).

For ultrastructural observations, specimens were cut in 1 mm^3 fragments and routinely fixed in 2.5% (v/v) glutaraldehyde (Agar Scientific, Stansted, UK) in Tyrode's buffer pH 7.2 (135 mM NaCl, 2.8 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 12 mM NaHCO$_3$, 0.4 mM NaH$_2$PO$_4$, 5.5 mM Glucose), postfixed in 1% (v/v) osmium tetroxide (Agar Scientific) in Tyrode's buffer, and dehydrated and embedded in Spurr resin (Agar Scientific). Ultrathin sections (approximately 70–80 nm) were stained with uranyl acetate and lead citrate and observed with a TEM Jeol 2010 (Jeol, Tokyo, Japan).

### MS-based techniques: the strategy

A workflow of the analytical approach and technologies we have combined in this study is presented in Fig. 2.

A typical experimental design for MALDI MS protein analysis is made of 3 steps: sample preparation, data acquisition (profiling and imaging) and data processing.

In the *profiling experiment*, the laser beam irradiates each sample spot and ion signals from hundreds of consecutive shots are averaged across the droplet surface generating a mass spectrum. Protein patterns from a discrete number of spots or areas can be compared allowing the analysis of molecules within their native environment.

For *imaging analyses* (MALDI IMS), spectra are recorded for each *x,y* coordinate into the mass range 2.5–30 kDa and finally plotted in 2-dimensions for ion density map construction (for each m/z value). Hundreds of protein-specific ion density maps correlated with tissue architecture can be generated. Each pixel (spectrum) contains many proteins and endogenous peptides that are individually displayed as a function of their position and relative intensity within the tissue. Spectra from different regions of interest (i.e. papillary, reticular and mineralized dermis) can be used for statistical analysis.

In a parallel experiment a histology-directed on-tissue protein digestion approach has been applied. Briefly, on-tissue protein digestion was performed using hydrogel discs (1 mm in diameter embedded with trypsin solution) placed on the regions of interest of cryosectioned samples. After digestion, discs were first manually removed from the tissue surface, then properly treated (solvent extracted) and used for LC–MS/MS analysis followed by database search for protein identification (ID).
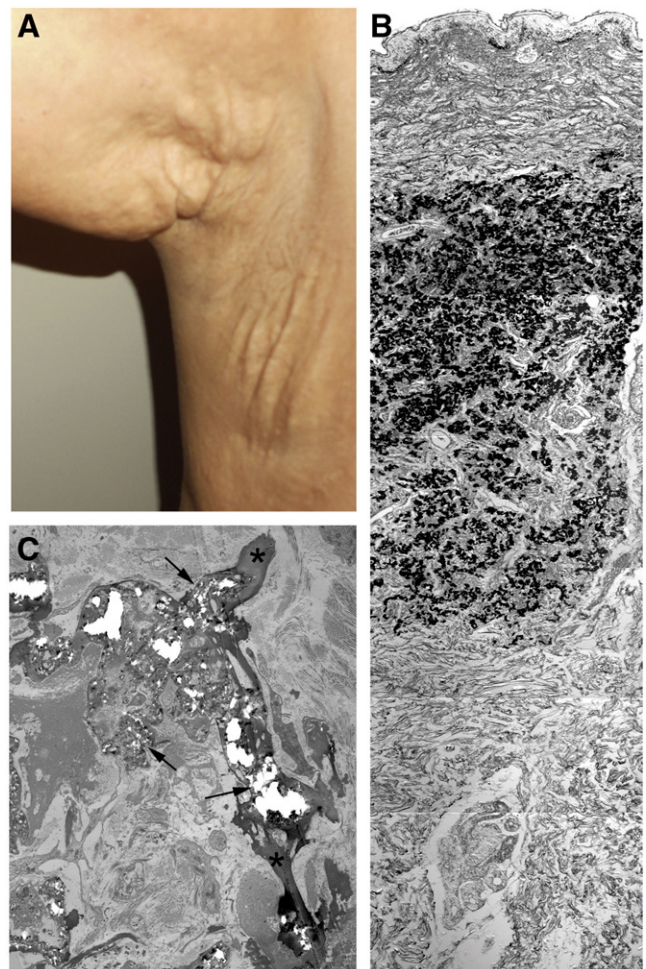
Fig. 1. Clinical phenotype and morphological features observed in PXE. (A–C) Skin laxity (A) is associated to the presence of extended areas of calcification in the reticular dermis as shown after von Kossa staining (B). By ultrastructural analyses (C) it appears that mineral deposits (arrows) are present within elastic fibers, thus altering their typical amorphous structure (asterisk).