Reflectance confocal microscopy and optical coherence tomography for the diagnosis of bullous pemphigoid and pemphigus and surrounding subclinical lesions

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Abstract

Background Diagnosis of bullous pemphigoid (BP) and pemphigus is based on clinical features, histology, immunofluorescence and laboratory data.

Objectives To evaluate features of BP and pemphigus at reflectance confocal microscopy (RCM) and optical coherence tomography (OCT) in order to provide a rapid non-invasive bed-side diagnosis. Secondary objective was to evaluate the detectability of clinically non-visible lesions.

Methods This was an observational, retrospective, multicentre study in which patients with suspicious lesions for BP or pemphigus underwent clinical assessment, RCM, OCT, blood tests and skin biopsy for histological and direct immunofluorescence examinations from January 2014 to December 2015. A total of 72 lesions in 24 selected patients were evaluated. Additionally, apparently unaffected skin at two different distances [near (1–2 cm) and far (2–3 cm)] from each lesion was examined to test subclinical lesion detectability.

Results RCM was able to detect subepidermal and intra-epidermal blisters, respectively, in 75% and 50% of the patients affected by BP and pemphigus. At OCT, the exact blister level was identified in all patients. Acantholytic cells were observed only at RCM in pemphigus (62.5%). Fibrin deposition inside the blisters was only found in BP, evidenced both at RCM and OCT. Among patients with BP, subclinical blisters were detected in nine (9.4%) clinically healthy skin, while among patients with pemphigus were observed in 10 (20.8%) apparently unaffected skin.

Conclusion RCM and/or OCT provide useful information for a rapid diagnosis of BP and pemphigus and for the identification of biopsy site. Combined use of RCM and OCT is optimal because associates the higher resolution of RCM with the greater penetration depth of OCT. OCT could be an optimal tool for treatment monitoring, especially in the cases of subclinical lesions. However, histopathologic and immunologic examinations remain the gold standard for establishing the final diagnosis.

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Conflicts of Interest

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Introduction

Bullous pemphigoid (BP) and pemphigus are chronic autoimmune diseases characterized by the presence of cutaneous and/or mucosal subepidermal and intra-epidermal blisters, respectively.1,2 BP is specifically characterized by the presence of autoantibodies specific for the hemidesmosomal bullous pemphigoid antigens BP230 (BPAg1) and BP180 (BPAg2).2,3 Upon histological examination, BP presents with subepidermal blistering with a striking inflammatory infiltrate predominantly consisting of eosinophils.1,2 Eosinophils are usually found within the blister as well as in the oedematous papillary dermis.1 Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are the two
main variants of pemphigus.\textsuperscript{1} PV is characterized by the presence of autoantibodies directed against desmoglein 3 (Dsg 3) and desmoglein 1 (Dsg 1), while only autoantibodies against Dsg 1 are identified in PF.\textsuperscript{2,3} PV is characterized histologically by the loss of adhesion among keratinocytes (acantholysis) with the develop of suprabasal bullae and by slight inflammatory infiltration of the upper dermis.\textsuperscript{4} The formation of PF blisters is more superficially in upper epidermis just below the stratum corneum.\textsuperscript{4} The diagnostic standard of care for BP and pemphigus is direct and indirect immunofluorescence, which displays IgG and C3 deposit at the dermoeidermal junction and in the epidermis, respectively.\textsuperscript{1,2} Enzyme-linked immunosorbent assay and immunoblot or immunoprecipitation analyses are confirmatory tests that are usually performed using recombinant proteins or keratinocyte extracts.\textsuperscript{1,4} However, these examinations are time-consuming and can delay the diagnosis. Reflectance confocal microscopy (RCM)\textsuperscript{5–11} and optical coherence tomography (OCT)\textsuperscript{10–12} are non-invasive imaging techniques that have been suggested for a rapid diagnosis of BP and pemphigus. The main objective of this study was to evaluate the capability to identify the exact location of the bulla, and other disease-related parameters detectable in epidermis and dermis by means of RCM and OCT in order to provide a rapid non-invasive bed-side diagnosis. Secondary objective was to evaluate the detectability of clinically non-visible lesions.

**Materials and methods**

**Study design**

This was an observational, retrospective study carried out in two institutions (University of Modena and Reggio Emilia, Italy and University Hospital of Saint-Étienne, France) from January 2014 to December 2015. Patients with clinically evident BP or pemphigus, who were sent directly for urgent biopsy, were excluded from the study. Patients with clinical suspicion of BP or pemphigus were sent for further non-invasive diagnostic examinations and were selected. The study was conducted according to the principles of the Declaration of Helsinki.\textsuperscript{13}

All patients ≥18 years old with suspicious BP or pemphigus lesions, who gave written informed consent, were considered for the study. Inclusion criteria also required the RCM and OCT examinations, histology, immunoblot analysis, direct and indirect immunofluorescence examinations. After clinical examination, three recent cutaneous lesions (erythematous scaly-to-crusted lesions, tense or flaccid blisters) per patient and clinically healthy skin at two different distances [surface area near (1–2 cm) and far (2–3 cm)] from each lesion were examined by RCM and OCT. The adjacent area (<1 cm) was not investigated due to the high risk of positive Nikolsky’s sign in patients with pemphigus. Two different investigators (V.D.M. and E.C.) examined together the RCM and OCT images. Following these initial evaluations, diagnosis of BP and pemphigus was all confirmed by histological examinations, along with immunoblot analysis, direct and indirect immunofluorescence.

**Features of bullous pemphigoid and pemphigus evaluated by RCM and OCT**

Considering the cleft formation, the presence of blister and its anatomical location (intra-epidermal, subepidermal or undefined level of the blister), inflammatory cells and fibrin deposition inside the bullae were evaluated by both RCM and OCT, whereas acantholytic cells were assessed only at RCM (Table 1). Moreover, the presence of disarranged epidermis, spongiosis and inflammatory cells in the epidermis was evaluated by RCM, whereas dilated blood vessels in the upper dermis were assessed by both RCM and OCT (Table 1).

**Imaging instruments**

A set of standardized clinical pictures were acquired on the three recent cutaneous lesions per patients with the Canfield Nikon D90 Digital SLR\textsuperscript{®} and the Canfield Close-up Scale\textsuperscript{®} (Canfield Imaging Systems, Fairfield, NJ, USA). RCM and OCT imaging were performed with RCM (VivaScope\textsuperscript{®} 3000: Mavig GmbH, Munich, Germany) and OCT (VivoSight\textsuperscript{®}: Michelson Diagnostics, Maidstone, UK) on the same cutaneous lesions per patient and on clinically healthy skin at two different distances from each lesion. RCM uses a diode laser with a wavelength of 830 nm and a power <22 mW at the tissue level; it scans an area of 1 × 1 mm up to a depth of 200 μm (upper dermis) and has a lateral resolution of approximately 1 μm and an axial resolution of 3–5 μm. OCT allows the horizontal visualization of the epidermis and the superficial dermis and the observation of cell morphology with a nearly histological resolution. The VivoSight\textsuperscript{®} OCT scanner uses the technique of swept-source OCT with a central wavelength of 1305 nm for cross-sectional imaging of the skin. It scans an area of 6 mm by 6 mm up to a depth of 2 mm, with a lateral resolution >7.5 μm and axial resolution >5 μm. Acquisition procedures have been described elsewhere.\textsuperscript{14,15}

**Statistical analysis**

Absolute and relative frequencies for RCM and OCT criteria were obtained. Statistical analyses were performed using the statistical package for social sciences statistical software (IBM\textsuperscript{®} SPSS Statistics\textsuperscript{®} for Windows, version 20.0: IBM Corporation, Armonk, NY, USA).

**Results**

In total, 72 lesions (48 BP lesions and 24 pemphigus lesions) from 24 patients (16 with BP and 8 with pemphigus) were evaluated (Figs. 1 and 2). Among the eight pemphigus patients, six were diagnosed with PV and two with PF. The median age of the patients was 70 years (range 28–92). Lesions were mainly located on the trunk and upper limbs (75%).
| Features                      | Description of observed features                                                                 | Incidence in bullous pemphigoid (N = 48) | Incidence in pemphigus (N = 24) |
|-------------------------------|-------------------------------------------------------------------------------------------------|-----------------------------------------|---------------------------------|
|                              | RCM                                                                                             | OCT                                     | RCM                            | OCT                            |
| Lesions                       |                                                                                                 |                                         |                                |
| **Presence of blisters**      | Dark ovoid areas corresponding on histology to the blisters                                      | 48 (100%)                               | 24 (100%)                      |
| (a) Intra-epidermal blister   | Intra-epidermal dark ovoid area with reappearance of epidermal layer (honeycomb) below the dark area (visible with VivaStack®) | 0 (0%)                                  | 12 (50%)                       |
| (b) Subepidermal blister      | Subepidermal dark ovoid area with appearance of dermal structures (collagen fibres) below the dark area (visible with VivaStack®) | 36 (75%)                                | 0 (0%)                         |
| (c) Undefined level of the blister | Dark ovoid area with non-assessable deep limit level                                             | 12 (25%)                                | 0 (0%)                         |
| Inflammatory cells inside the blisters | Small (8–12 μm diameter), mildly bright cells, round to polygonal in shape with no visible nucleus inside the blisters | 30 (62.5%)                              | 6 (25%)                        |
| Fibrin deposition inside the blisters | Mildly refractive homogeneous material inside the blisters                                      | 30 (62.5%)                              | 0 (0%)                         |
| Acantholytic cells            | Big (14–17 μm diameter), weakly refractive round or ovoid cells with centrally or eccentrically situated nucleus, often arranged in clusters, forming irregular polygonal structures | N/A                                      | 15 (62.5%)                     |
| Epidermis                     |                                                                                                 |                                         |                                |
| Disarranged epidermis         | Partial or complete loss of regular honeycomb pattern of epidermis                              | N/A                                     | 18 (75%)                       |
| Spongiosis                    | Darker areas among keratinocytes in the stratum spinosum of the epidermis                       | N/A                                     | 6 (25%)                        |
| Inflammatory cells in the epidermis | Small (8–12 μm diameter), highly refractive cells, round to polygonal in shape with no visible nucleus in the epidermis | N/A                                     | 15 (62.5%)                     |
| Dermis                        |                                                                                                 |                                         |                                |
| Dilated blood vessels in the upper dermis | Prominent round or linear dark structures within upper dermis                                | 21 (43.8%)                              | 18 (75%)                       |

N/A, not applicable.
Blister features of bullous pemphigoid and pemphigus

Blisters were detectable in all cases, while a precise assessment at the subepidermal and intra-epidermal location was possible in 75% of BP and 50% of pemphigus, respectively (Table 1). Disarranged epidermis, acantholytic cells and intra-epidermal blisters were found in more than half of pemphigus patients,
but were undetected in patients with BP, whereas subepidermal blisters and fibrin deposition inside the blisters were evident in the majority of BP patients, but not in patients with pemphigus. The inflammatory cells in the epidermis, the dilated blood vessels in the upper dermis and the undefined blister level were more frequently observed among patients with pemphigus compared with BP. Inversely, inflammatory cells inside the blisters were more evident in patients with BP. Spongiosis was equally detected in patients with BP and pemphigus.

**Figure 3** RCM (a and b) and OCT (c) executed on the clinically healthy skin of patients affected by BP and pemphigus generally showed normal layer architecture. S: stratum corneum; E: epidermis; J: dermoepidermal junction; H: hair.

**Figure 4** OCT performed on the clinically healthy skin of patients with BP revealed in the surface area near (a) and far (b) from the lesion: the presence of subclinical clefts (purple arrows), inflammatory cells (green arrow) and fibrin deposition (blue arrows) inside the blisters. OCT executed on the clinically healthy skin of patients affected by pemphigus showed in the surface area near (c) and far (d) from the lesion: the presence of subclinical clefts (purple arrows). S: stratum corneum; E: epidermis; J: dermoepidermal junction; H: hair.

**OCT features of bullous pemphigoid and pemphigus**

Blisters were identified in all cases of BP and pemphigus, at the intra-epidermal level for all patients with pemphigus and at the subepidermal level for all patients with BP (Table 1). Inflammatory cells inside the blisters were more frequently detected in patients with BP than in patients with pemphigus (62.5% vs. 25% respectively). Fibrin deposition inside the blisters was detected in the majority (62.5%) of BP patients only. Dilated blood vessels in the upper dermis were equally found in most patients with BP and pemphigus (68.5% and 75%, respectively).
RCM and OCT examination of the clinically healthy skin of patients with bullous pemphigoid and pemphigus

A total of 144 clinically healthy skins were examined: 96 and 48 images of apparently unaffected skin acquired with RCM and OCT from patients with BP and pemphigus, respectively. These instrumental examinations generally showed normal skin architecture (Fig. 3), but subclinical bullae were revealed by OCT in some patients affected by BP and pemphigus (Fig. 4). Among patients with BP, subclinical blisters were detected in 9 (9.4%) clinically healthy skin [four and five in the surface area near (8.3%) and far (10.4%) from the lesions, respectively], while among patients with pemphigus were observed in 10 (20.8%) apparently unaffected skin [seven and three in the surface area near (29.2%) and far (12.5%) from the lesions, respectively].

Discussion

It is not always possible to diagnose exactly an autoimmune bullous disease only on the basis of the clinical findings due to the frequent heterogeneous clinical features. Therefore, currently the diagnosis of BP and pemphigus is based on a combination of clinical, histological, immunofluorescence and laboratory data. However, these examinations are time-consuming and not without limitations and may delay the diagnosis and the appropriate therapy that is essential to prevent a severe and potentially life-threatening course. The biopsy for histopathology is an invasive procedure and can be non-diagnostic while the immunofluorescence can provide false-negative results for sampling and/or technical errors. In fact, the biopsy of blistering diseases is notoriously hampered by the difficulty in obtaining representative samples of the blisters because of their possible rupture following even minimal manipulation during the biopsy procedure. In contrast, RCM and/or OCT permit direct visualization of the blister tissue and the non-invasive nature of these procedures, as opposed to conventional biopsies, they easily allow the examination of multiple lesions in the same patient, thereby increasing the probability of obtaining representative samples. The possibility of a real-time evaluation with RCM and/or OCT to support the clinical diagnosis and better orient the clinician is thus interesting for early, tailored patient management, and an optimum biopsy site selection. To the best of our knowledge, the use of RCM and OCT for the diagnosis of BP and pemphigus has generally been reported separately. Levi et al. demonstrated the usefulness of RCM in determining the blister level in all eight patients (two with PF, three with PV and three with BP) enrolled in the study. Kurzeja et al. described the characteristic RCM features of pemphigus in 30 patients (18 with PV and 12 with PF) and proposed three RCM criteria for diagnosing pemphigus (acantholytic clefts in RCM of healthy-appearing skin adjacent to a lesion, acantholytic clefts and multiple dilated blood vessels in RCM of the lesion). In detail, RCM was able to visualize intra-epidermal clefts with acantholytic cells in 47% of PV and 59% of PF lesions (predominantly in the peripheral portion of these lesions and most commonly in recent lesions) and multiple dilated blood vessels in the upper dermis in 61% of PV and in 86% of PF lesions. The authors concluded that the identification of the exact intra-epidermal localization of the clefts may be difficult in some cases, especially when the structure of the epidermis is uneven and wavy or a concomitant disarrangement of epidermal layers is present. Therefore, the blister level can be determined using RCM, but this instrument may not be considered sufficiently precise for differentiating PV from PF in clinical practice. Ardigo et al. described the characteristic RCM features of BP in seven patients. The authors reported that the presence of subepidermal cleft, which was identified by RCM in all the bullous lesions, and a variable amount of oedema of the upper dermis associated with inflammatory cells infiltration was seen as prevalent confocal features of BP. Instead, Mogensen et al. explored the potential role of OCT in bullous skin disorders in a small patient cohort (three with BP, one with extensive bullae following burns, one with pemphigus, one with subcorneal pustular dermatosis and one with Darier’s disease). The authors suggested that OCT images may match histopathology regarding the level and the architecture of the blisters in bullous diseases. In our study, we evaluated the capability to identify the exact location of the bulla and other disease-related parameters detectable in epidermis and dermis by means of RCM and OCT, showing how these two technologies work in a complementary way. Both RCM and OCT enabled the visualization of the blisters as dark subepidermal or intra-epidermal spaces in all patients affected by BP and pemphigus, respectively. RCM, providing horizontal images and a series of horizontal ‘cuts’ at various depths (z-stacks), can determine the level and full extension of the blister. However, it was not always possible to identify with certainty the location of the clefts (undefined level of the blister) in twelve of BP and pemphigus lesions (25% and 50%, respectively) at RCM. These undefined blisters at RCM appeared such as dark ovoid area with non-assessable deep limit level. Large blisters, inflammatory phenomena and laser power attenuation can render the junctional transition difficult to distinguish in some occasions. Therefore, it is important to evaluate more than one lesion with RCM and to select small blisters in case of BP. Conversely, OCT allowed an improved visualization of the blister level compared to RCM, mainly due to its higher penetration depth (up to 2 mm) and the cross-sectional images of the skin, but with a lower resolution. In particular, OCT was also able to show large BP blisters in their entire extension and to identify the exact location of the epidermal cleft in pemphigus. Moreover, in proximity to some BP and pemphigus lesions, small subepidermal and intra-epidermal bullae were, respectively, observed by means of OCT examination, not visible at clinical examination. This anticipated detection of lesions offers the unique opportunity to monitor treatment and identify disease relapse prior to clinical evidence. Concerning the other features.
evaluated in our study, RCM and OCT gave similar results except for dilated blood vessels. Dilated blood vessels in the upper dermis were found more often in pemphigus than in BP, and this difference was more marked at RCM than OCT imaging, probably due to the lower RCM ability to observe the deep vessels beneath large BP blisters. Interestingly, fibrin deposition inside the blisters was visible both at RCM and OCT imaging in most BP but not in any pemphigus. Moreover, inflammatory cells inside the blisters were more common in BP than in pemphigus, evident at both RCM and OCT. Disarranged epidermis and acantholytic cells were additional features that differentiated BP from pemphigus, present in the majority of pemphigus, but not in BP. Inflammatory cells in the epidermis were more frequent in pemphigus than in BP but were not exclusive of pemphigus. Spongiosis was found in the same extent in a small proportion of BP and pemphigus. When examining bullous lesions with RCM and OCT, the strengths and weaknesses of both technologies should be taken into account. OCT seems very suitable for a precise definition of the bullae location and for the detection of subclinical lesions (thus, indicated for BP vs. pemphigus differential diagnosis and treatment monitoring). However, some microscopic parameters could not be detected by OCT, due mainly to the lower resolution compared to RCM, such as disarranged epidermis, acantholytic cells, spongiosis and inflammatory infiltrate in the epidermis, which may be useful in differential diagnosis between autoimmune blister diseases (such as BP and pemphigus) vs. other bullous disorders (such as viral bullae like in varicella or other blistering viral infections).17–20 However, although determination of the blister level and other features with RCM and OCT is directly helpful in the work-up of these patients, it should be noted that at present, these instruments cannot replace histology, laboratory data and immunofluorescence, which are still required for diagnosis confirmation.

Conclusions

RCM and/or OCT offer useful information for a rapid non-invasive diagnosis of bullous disease and for the identification of biopsy site, which can aid pathology evaluation and save time ahead of a confirmatory diagnosis, anticipating treatment and improving patient management. Combined use of RCM and OCT for a real-time examination of the skin lesions is optimal because it associates the higher resolution of RCM with the greater penetration depth in cross-sectional view of OCT, providing in vivo quasi-histological information. However, according to clinical requirements, RCM and OCT could be employed alternatively to satisfy different clinical questions. In particular, OCT allows a clear definition of the anatomical location of the bulla and enables the identification of subclinical lesions, while RCM detects some microscopic parameters useful for the differential diagnosis of vesicobullous diseases.

Consent

Written informed consent was obtained from the patients for publication of this manuscript and accompanying images.

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