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ORIGINAL ARTICLE

Angiogenesis in Oral Lichen Planus: An In Vivo and Immunohistological Evaluation

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Abstract Oral lichen planus (OLP) is an autoimmune disease with an inflammatory pathogenesis. The angiogenetic phenomenon is a mechanism at the base of the pathogenesis of chronic inflammatory processes. The aim of this research is to evaluate the angiogenetic phenomenon, comparing an in vitro method with an in vivo one. Thirty OLP patients and 30 healthy subjects were enrolled in the study. Immunohistochemical analysis of the vascular-endothelial growth factor (VEGF) and vascular-endothelial adhesion molecules were carried out by the means of primary antibodies and anti-CD34, anti-VEGF, anti-CD106 antigen (VCAM-1) and anti-CD54 antigen (ICAM-1). Capillary density and others capillaroscopic parameters were tested in vivo using oral videocapillaroscopy. The results reveal the presence of a significant angiogenesis in OLP patients through the immunoexpression of VEGF, CD34, CD106 and CD54 ($p < 0.001$). Capillaroscopic analysis demonstrates significant value for the following parameters: density, tortuosity, loop diameter, afferent and efferent capillary loop diameter. The in vivo and in vitro investigation in OLP reveals a significant angiogenesis.

Keywords Angiogenesis · Videocapillaroscopy · VEGF · Immunohistochemistry

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Introduction

Lichen planus is a chronic inflammatory autoimmune disease of unknown aetiology. It affects predominantly the oral mucosa but can involve the skin and other mucosal sites such as the genital region. The incidence of the pathology is greater in women aged between 30 and 60 (Bermejo-Fenoll et al. 2010; Farhi and Dupin 2010). There is a debate in literature on the possibility of the neoplastic transformation of oral lichen planus (OLP). All the authors agree on the necessity of a meticulous follow-up of the lesion to carry out a precocious diagnosis and eliminate all the possible risk factors (Obradović et al. 2009; Torrente-Castells et al. 2010). Regarding this matter, van der Waal sustains that there is an ongoing debate in the literature whether the patients with OLP carry an increased risk of developing a squamous cell carcinoma (SCC). Apparently, such events may occur in all clinical types of OLP. Unfortunately, the issue of malignant transformation in OLP is blurred by the often present lack of clinicopathologic correlation in the diagnosis (van der Waal 2010).

The role of angiogenesis in the pathogenesis of chronic inflammatory diseases is very important.

Angiogenesis represents a cycle of vital processes that lead to the formation of new blood vessels starting from pre-existing vascular structures. The fulcrum of angiogenesis is the endothelial cell that proliferates and differentiates under the direct and indirect regulatory action of vascular-endothelial growth factor (VEGF), the principal direct inducer of angiogenesis. The angiogenetic process plays an important role both in physiological conditions and pathological ones. The normal growth of the tissue, the development of the embryo, the healing of wounds and the menstrual cycle are physiological conditions characterised by the formation of new vessels for supplying O_2 and nutritive substances and

for the elimination of metabolic residues. Instead, from a pathological point of view, angiogenesis is implicated in the development of neoplasia and metastasis and in the ethiopathogenesis of chronic inflammatory pathologies such as rheumatoid arthritis, psoriasis, bronchial asthma, diabetic retinopathy, atherosclerosis, Alzheimer's disease, Crohn's disease and ulcerative rectocolitis. Investigations have shown a close relationship between angiogenesis and the activity of these diseases and it may be one of underlying markers of disease activity of OLP (Murphy et al. 2010).

The aim of this research is to evaluate the angiogenetic phenomenon in OLP, comparing an in vitro method with an in vivo one.

Materials and Methods

Thirty OLP patients (M/F: 24/6; mean ± SD: 54.8 ± 0.23) and 30 healthy volunteer subjects (M/F: 22/8; mean ± SD: 52.5 ± 0.57) were enrolled in the study. Volunteers were fully informed as to the risks and benefits of the procedure and they could withdraw from the study at any time. Students and junior staff who had volunteered were excluded. The diagnosis of OLP was performed according to the WHO diagnostic criteria for oral lichen planus. These criteria are based on the clinical and histopathological characteristics. Clinical criteria are: presence of bilateral, more or less symmetrical lesions; presence of a lacelike network of slightly raised grey white lines (reticular pattern); erosive, atrophic, bullous and plaque-type lesions are only accepted as a subtype in the presence of reticular lesions elsewhere in the oral mucosa. Histopathological criteria are: presence of a well-defined band-like zone of cellular infiltration that is confined to the superficial part of the connective tissue, consisting mainly of lymphocytes; signs of 'liquefaction degeneration' in the basal cell layer; absence of epithelial dysplasia (van der Meij et al. 2007).

Two specialist oral histopathologists, blinded to the source of the biopsy material made the histopathological diagnosis of OLP and the assessment of the immune-histochemical staining.

Every patient which had lichenoid lesions objectively related to exogenic agents such as dental materials, drugs or specific allergens were excluded from the sample group (Thornhill et al. 2006). The study received the approval from the ethical commission of the University Hospital "Paolo Giaccone" of Palermo (No. 02/2010, assembly 03 February 2010).

The biopsy sample from subjects suffering from OLP was taken from lesion areas to better inspect the histological characteristics of the pathology. In the healthy subjects included in the study, the biopsy sample was taken from the same corresponding area as the lesion in OLP patients. All the healthy subjects employed in the study were not affected by any systemic pathology, did not take any drug nor had any bad habits like alcohol or smoking. All the patients of the case study group and the control group gave their informed consent in accordance with Italian law and as established by the ethics commission.

Histological and Immunohistochemical Investigation

After having signed the informed consent the patients and the healthy subjects were submitted to an incisional biopsy through means of a biopsy punch (ø = 4 mm). All the biopsy samples were analysed using the same procedures and materials. For each sample, embedded in paraffin after being fixed in formalin, four sections of tissue around 5 μm thick were prepared to enable an immunohistochemical analysis of the VEGF and vascular-endothelial adhesion molecules in the vascular endothelial cells. These sections were treated with primary antibodies (Dako and Novocastra Lab, New Castle upon Tyne, United Kingdom #7071) anti-CD34, anti-VEGF, anti-CD106 antigen (VCAM-1), anti-CD54 antigen (ICAM-1), anti-UCHL1 (ubiquitin carboxyl-terminal hydrolase L1) and periodic acid Schiff stain (PAS). The dilution of the primary antibodies was: 1:500 for anti-VEGF and 1:50 for anti-CD106, anti-CD34, anti CD-54, anti-UCHL1 and PAS. Positive and negative immunohistochemical controls for the different antibodies used were stained in parallel with the studied sections.

The primary antibodies were revealed with the standard avidin–biotin method. Angiogenesis was valuated with a semiquantitative method counting for all the markers and in every histological section and in five random fields with high magnification (400×).

Videocapillaroscopic Investigation

Oral videocapillaroscopy is a simple, repeatable, non-invasive, panoramic technique that is performed in vivo and is well-tolerated by the patient. This computerised technique is used with specific software (DS Medigroup, Milan, Italia) that allows the acquisition and the elaboration of the data. The Videocapillaroscope is made up of a central unit, a fibre optic probe with video-optic terminal and a high resolution colour monitor. The lens has a focal spot of 1.811 mm and enlargements which vary from 10 to 1,000×. For the study in question, a 200× enlargement was used. The morphofunctional evaluation of microcirculation is based on specific parameters: length of the capillary loop, diameter of the loop, diameter of afferent and efferent loops, capillary tortuousness and density. The videocapillaroscopic investigation was always performed with the same light source, at the same room temperature (23°C), in

the morning, with the same operator (GAS) and repeated many times for every area under investigation (Mărgăritescu et al. 2010). The site subjected to videocapillaroscopic investigation was the same site subsequently biopsied. This correlation was achieved in practice using a dermoscopic pencil to highlight the area under observation, which had a diameter of about 2 centimetres.

The following static parameters were analysed:

Non-Parametric Data

- Capillary loop visibility (graded 1–4): (1) simple focusing—within 30 s from the beginning of the examination; (2) average focusing—over 30 s and within 2 min; (3) difficult focusing—over 2 min; (4) impossible focusing;
- Orientation to the surface (graded A, B or AB): (A) capillary loop course parallel to the surface; (B) capillary loop course perpendicular to the surface; (AB) both parallel and perpendicular;
- Microhemorrhages (graded 0 or 1): (0) absence; (1) presence;
- Characteristics of the capillary loops (graded 0 or 1): (0) absence; (1) presence.

Parametric Data:

- Capillary length;
- Capillary density (number of visible capillary loops per square millimetre—value obtained from the average of the two observations for examined area);
- Capillary loop diameter (total, afferent and efferent loop);
- Tortuosity: number of crossing in the capillary loop (value obtained from the average of the two observations for examined area) (Scardina et al. 2009a, b, c).

Statistical Analysis

The statistical significance of the differences was checked with the Mann–Whitney test. The level of significance was set to $p < 0.001$. Data analysis was carried out with StatView 5.0.1 (SAS Institute Inc., Cary, NC).

Results

The age difference between the patients affected by OLP and the healthy subjects was not statistically relevant.

The number of vessels in the biopsies of the patients with OLP (mean ± SD: 21.27 ± 4.85), compared with the healthy subjects (mean ± SD: 4.74 ± 0.97) was significantly increased (Mann–Whitney test, $p < 0.001$). The positive expression rate of VEGF, CD34, VCAM-1, ICAM-1, UCHL1 and PAS in oral lichen samples were 64.2, 54.3, 32.5, 29.7, 28.4 and 31.5%, respectively (Table 1). Moreover in the context of the inflammatory process and in a random way, both isolated endothelial cells immunopositive to the antibody anti-VEGF and neoformed microvessels and endothelial cells with high-immune-positivity to the antibodies anti-ICAM-1 and anti-VCAM-1 were observed (Figs. 1, 2). In healthy subjects, we had a higher positive percentage for CD34 and an extremely low percentage for VEGF, ICAM, VCAM, UCHL1 and PAS.

Videocapillaroscopic investigation produced significant data. Capillary density and the diameter of the afferent and efferent ansae were found to be significantly increased in OLP patients compared with controls. There was also a significant difference between the study of capillary tortuosity and the discovery of characteristic branched loops, indicating angiogenesis (Table 2).

Discussion

Angiogenesis is a fundamental process in the pathogenesis of chronic inflammatory pathologies and contemporarily represents the base of the activity of OLP. For this reason the angiogenetic phenomenon and its markers, above all VEGF and the molecules of endothelial adhesion, have been the object of many recent studies (El-Rouby 2010; Margaritescu et al. 2010; Murphy et al. 2010; Okada et al. 2010; Schaaij-Visser et al. 2010; Williams 2010).

OLP as a chronic inflammatory pathology that finds in angiogenesis and in the variation of the capillary density a critical point that needs particular attention.

OLP, like any analogous chronic inflammatory disease, has significant secondary angiogenesis in response to the hypoxic effect on the inflamed stromal area caused by an increase of the proliferating lymphocitary cellular mass. This feed-back mechanism is evidenced by the presence of isolated endothelial cells which stimulate endothelial-vascular proliferation (VEGF). The lymphocytes in transit between the cubic endothelial cells of newly-formed vessels suggest that angiogenesis in the lichen not only restores tissue oxygenation but also has an important role in the turnover of inflammatory elements in the inflamed area and thus in the self-maintenance of the illness.

Since angiogenesis has been found to contribute to pathogenesis and to correlate closely with the disease activity of a series of chronic inflammatory conditions, including rheumatoid arthritis, psoriasis, and osteoarthritis, the result of our investigation suggest that angiogenesis may play a role in the chronicity of OLP and may be a marker of disease activity (Murphy et al. 2010).

Hussein (2007) has studied the cutaneous lichen from an immunohistochemical point of view with the aim of finding

Table 1 Conclusive results of the statistical analysis of immunohistochemical investigation performed by Mann–Whitney *U* test ($p < 0.001$)

	OLP patients	Healthy subjects
Age (mean ± SD)	54.8 ± 0.23	52.5 ± 0.57
Gender	24/F	22/F
	6/M	8/M
Number of vessels (mean ± SD)	21.27 ± 4.85	4.74 ± 0.97
Positive expression rate of markers (%)		
VEGF	64.2	4
CD34	54.3	18.3
CD106 (VCAM-1)	32.5	5
CD54 (ICAM-1)	29.7	7
UCHL1	28.4	6
PAS	31.5	7
Statistical significance (Mann–Whitney *U* test)	$p < 0.001$ significant values for VEGF, CD34, VCAM-1, ICAM-1, UCHL1 and PAS	

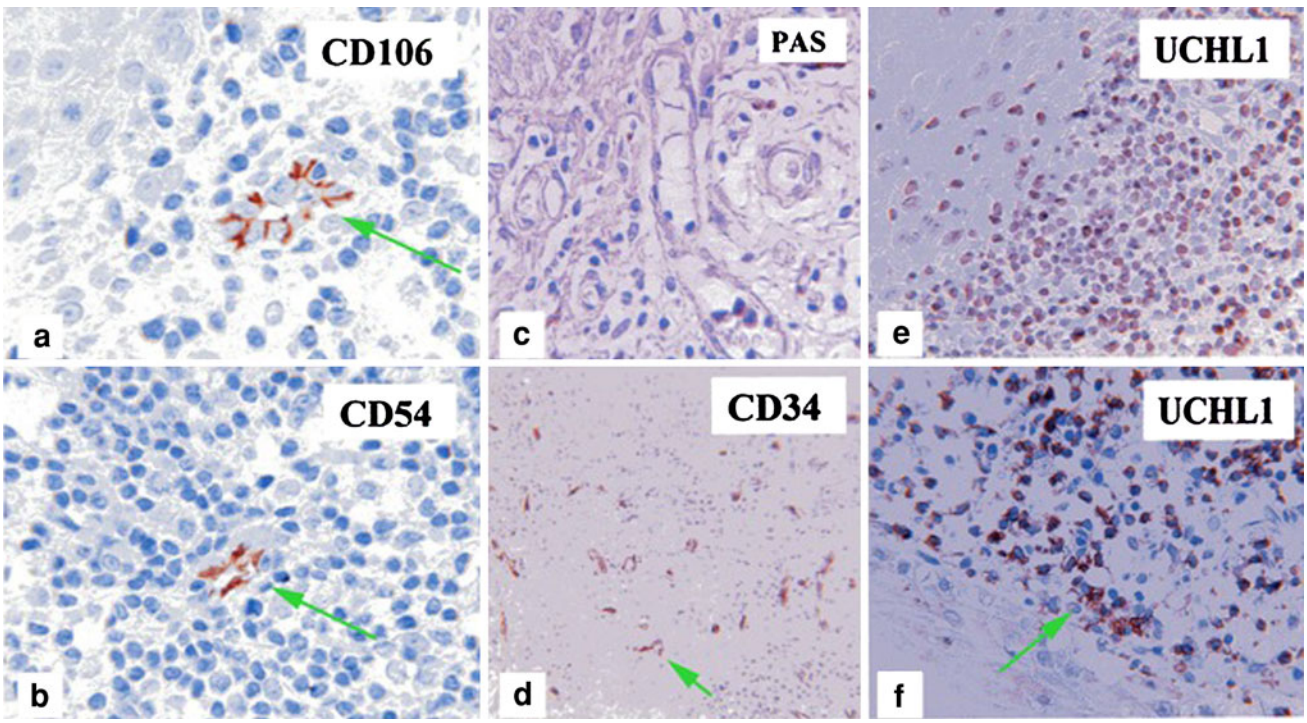

Fig. 1 Immunohistochemistry for microvessels in OLP patients: marked angiogenesis within lymphocytic infiltration. These sections were treated with primary antibodies anti-CD106 antigen (VCAM-1) (**a**), anti-CD54 antigen (ICAM-1) (**b**), PAS (periodic acid Schiff stain) (**c**), anti-CD34 (**d**), anti-UCHL1 (ubiquitin carboxyl-terminal hydrolase L1) (**e**, **f**). Magnification: ×400

alterations of dermal vascularization (microvessel density—MVD) immunostained for CD34 (angiogenetic marker). The higher MVD values in cutaneous lichen planus suggest a possible link between angiogenesis, and development of these cutaneous lesions. These results provide a morphological evidence of potent angiogenic activity in cutaneous lichen planus, indicating that the local microvasculature may undergo an intense process of inflammation-dependent angiogenesis (Hussein 2007).

Raspollini et al. (2007) has conducted a similar study on vulvar lichen sclerosus (LS). The MVD, and the expression of VEGF and of COX-2 were analysed in this study. Difference of VEGF and COX-2 expression between unchanged LS cases and LS cases evolving to SCC and LS adjacent to SCC cases were statistically significant. These results address the possibility that immunohistochemical studies may add information to permit the identification of LS as a precursor lesion (Raspollini et al. 2007). Tao et al.

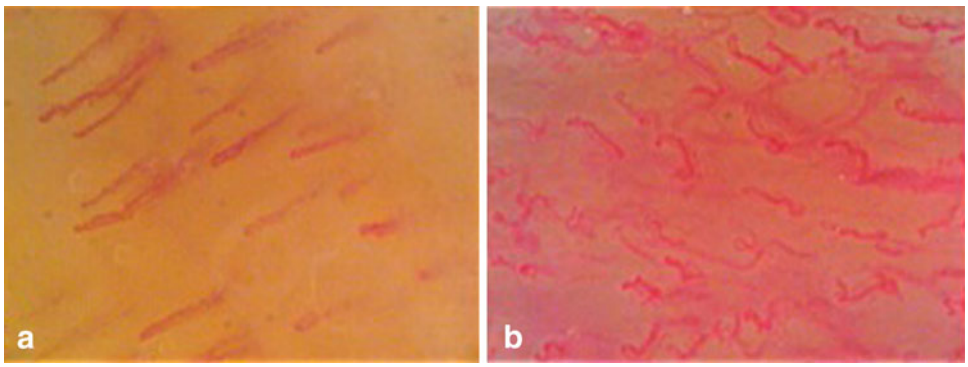

Fig. 2 Videocapilaroscopy in OLP and healthy subject: capillary density and the diameter of the afferent and efferent ansae were found to be significantly increased in OLP patients (**b**) compared with controls (**a**). Magnification: ×200

Table 2 Results of capillary parameters obtained using videocapillaroscopic examination performed by Mann–Whitney *U* test ($p < 0.001$)

	LPO patients	Healthy subjects	Significance
Capillary density	25.75 ± 16.13	21.17 ± 7.08	S
Capillary loop diameter	0.031 ± 0.01	0.026 ± 0.009	S
Afferent loop diameter	0.011 ± 0.004	0.006 ± 0.002	S
Efferent loop diameter	0.014 ± 0.003	0.008 ± 0.002	S
Loop length	0.20 ± 0.056	0.187 ± 0.06	NS
Tortuosity	2.21 ± 0.40	1.098 ± 0.80	S

S significant, *NS* not significant

(2007) assessed local angiogenesis in atrophic-erosive and reticular OLP using immunohistochemical evaluation. MVD in atrophic-erosive OLP was significantly higher than that in controls and reticular OLP, and the expression of VEGF in both subgroups was significantly higher compared to control group. Results indicated that angiogenesis and VEGF expression were closely correlated to different clinical forms of OLP lesions, which may give new insights into the mechanisms and treatment strategy of OLP (Tao et al. 2007). Scardina et al. (2009a) have studied the angiogenetic phenomenon in OLP patients using immunohistochemical analysis of the VEGF and vascular-endothelial adhesion molecules as anti-CD34, anti-VEGF, anti-CD106 antigen (VCAM-1) and anti-CD54 antigen (ICAM-1). Isolated endothelial cells and newly-formed micro-vessels and endothelial cells with high-immune-positivity to the antibodies anti-ICAM-1 and anti-VCAM-1 were observed (Scardina et al. 2009a, b). Scardina et al. have analysed in vivo using videocapillaroscopy all the vascular systems of the oral mucosa or the labial, lingual, buccal and parodontal mucosa. All the data gathered in roughly 10 years of studies on the variations of capillary density in the oral sites agree and attest to a significant variation of the capillary density as a translation of an effective angiogenetic phenomenon in OLP (Scardina et al. 2007a, b, 2009b, c).

In our research we observed a low expression of VEGF, ICAM, VCAM, UCHL1 and PAS in healthy subjects. Other research observed that the staining pattern of VEGF was similar among the atrophic-erosive, reticular, and control groups, whereas when all OLP patients were included as a group, the level of VEGF in OLP subjects was statistically greater. The results of our immunohistochemical research show that a significant angiogenesis occurs in OLP.

The comparison of the in vivo and the histological and immunohistochemical methods has led to interesting results by testing the effective variation in the angiogenesis in OLP and revealing a satisfactory parallelism between the two methods of investigation. The debate regarding the correlation between angiogenesis and OLP focuses on how much the phenomenon is due to the chronic inflammatory mechanism and on how much the increase of the capillary density found in the lichen both through immunohistochemistry and through the use of oral videocapillaroscopy can be considered a sign of activity of the disease. The screening performed through oral capillaroscopy is more easily repeatable, immediate, less invasive and less expensive respect to immunohistochemical investigation through the collection of a biopsy sample. The histological and immunohistochemical technique, however, surely brings more complete results since the histological investigation studies the tissue activity entirely while capillaroscopy focuses entirely on the vascular component. In light of such reflections, the authors suggest that in the protocols of follow-up, initial screening of capillary density should be performed by means of capillaroscopy to quickly individualise an abnormal alteration of the microcirculation. Detection of angiogenesis in vivo may be regarded as an indicator of angiogenesis in OLP. Therefore, our data strongly suggest that the in vivo evaluation may be useful and convenient as a translational marker in monitoring disease activity and therapeutical response. In fact the importance of angiogenesis in OLP has led to the application of anti-angiogenic treatment in patients who were

corticosteroid-resistant or contraindicated to systemic corticosteroids.

In this way the number of biopsy samples would concretely be reduced with a smaller waste of sanitary resources and greater comfort for the patient, who will not have to necessarily undergo histological investigation and can be quickly reassured on the static nature of the lesion or informed of possible modifications that need a more precise investigation.

In conclusion, the in vivo and in vitro investigation using immunohistochemical and capillaroscopy method reveals a significant angiogenesis in OLP.

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