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ORIGINAL PAPER

A study of the pathogenesis of Rosacea: how angiogenesis and mast cells may participate in a complex multifactorial process

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Abstract In the present study we evaluated, in involved and clinically uninvolved skin of Rosacea, microvessels density (MVD) and total vascular area (TVA) in addition to multiple morphologic characteristics of microvessels and also mast cells (MCs) number. We examined also the relationship between angiogenesis, MCs number and disease clinicopathological data. The study included 69 patients with Rosacea. A skin biopsy with a 4-mm punch was performed from clinically involved skin in each case. In nine randomly selected patients, facial biopsy specimens were obtained from both involved and clinically uninvolved skin. Histological sections, immunostained for factor VIII, were evaluated by image analysis for the quantification of MVD, TVA and several morphometric parameters related to the vessel size or shape. MCs detection in the dermis was carried out using the chloracetate esterase method (Fast Blue RR) in parafin sections. Serum antibodies against H.pylori were detected. Statistically important differences concerning the factors of angiogenesis between lesional and clinically non-lesional skin were demonstrated. A statistical important correlation was found also between high vascular density, PPR clinical type and the presence of ocular manifestations. MVD or TVA showed no correlation with the degree of solar elastosis or inflammation and with the Demodex density as well. However, high MVD values were found to correlate with granuloma formation in the dermis. MCs number were significantly greater in lesional compared to clinically non-lesional skin. Statistical significance was shown between MCs density and disease duration. However, no correlation between MCs number and blood vessel density was found. Angiogenesis seems to play an important role in the pathogenesis especially of the more severe clinical form of Rosacea. MCs seem to participate in evolution to disease chronicity by contributing to inflammation, angiogenesis and tissue fibrosis.

Keywords Rosacea · Angiogenesis · Mast cells

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Introduction

Rosacea is a chronic cutaneous disorder primarily of the convexities of the central face, often characterized by remissions and exacerbations. It produces a variety of clinical presentations, which are grouped into four subtypes as erythematotelangiectatic (ETR), papulopustular (PPR), phymatous and ocular. A progression from one subtype to another may or may not occur. However, each individual characteristic may progress from mild to moderate or severe [2, 12, 35].

The cause of Rosacea remains unknown. Several factors have been implicated in its pathogenesis, such as climatic exposure, vasculature, dermal matrix degeneration, chemical and ingested agents, pilosebaceous unit abnormalities, genetic susceptibility, microbial organisms (Demodex, *H.pylori*) [5, 21].

Recently, much research has focused on angiogenesis as an essential process in a variety of chronic inflammatory skin diseases such as psoriasis and bullous skin diseases [7, 10]. MCs have also been implicated as contributing agents to a number of inflammatory skin disorders by promoting localized vasodilatation, angiogenesis and tissue fibrosis [29, 34]. Both these factors have been speculated to contribute to the pathogenesis of Rosacea [1, 5].

In the present study we evaluated microvessels density (MVD) and total vascular area (TVA) in addition to multiple morphologic characteristics of microvessels in Rosacea and examined the relation between angiogenesis, MCs number and clinicopathological data. The evaluation of these parameters served to clarify certain steps in the evolution of Rosacea.

Materials and methods

A total of 69 randomly selected patients with Rosacea were examined (with a mean age of 47 ± 14.0 SD). Rosacea diagnosis was based on clinical evaluation and histological findings.

A skin biopsy with a 4-mm punch was performed in each case from clinically involved skin. In nine randomly selected patients, facial biopsy specimens were obtained from both involved and clinically uninvolved skin. The latter was biopsied at a point at least 5 cm from the edge of the nearest lesion.

Specimens were routinely processed, first fixed in neutral formalin and then stained with H-E, orcein-shikata for elastic fibers and Giemsa for mast cells. In addition the chloracetate esterase (Fast Blue RR) method in paraffin sections was used for the detection of MCs in the dermis [6].

Multiple sections H-E on each slide (20 serial sections on average) were examined under light microscopy. Histological data as solar elastosis and inflammation were evaluated semiquantitatively (mild +, moderate ++, severe +++).

Demodex mite presence was estimated semiquantitatively under light microscopy as follows: 0–1+, 2++, ≥3+++.

Serological antibodies (IgG and IgA) against *H.pylori* were detected by means of an enzymatic immunoabsorption test (enzyme-linked immunosorbent assay, ELISA, Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Endothelial cells were visualized using a rabbit polyclonal antibody against factor VIII (FVIII) (Dako Co., Carpinteria, CA, USA).

Evaluation of MVD and image analysis

Slides were examined carefully at low power magnification (40×) to identify the areas with the highest density of capillaries and small vessels (hot spots). In each case the most vascularized area was selected and a 200× field was photographed using a Zeiss Axioscop-microscope (Zeiss Inc., Thornwood, NY, USA) and printed on high-quality photographic paper. Photographs were scanned to become digital and were stored as JPEG files (1,550 × 1,070 pixels, 16.7 million colors [24-bi[T1]t]) (Fig. 1a). In cases in which the most vascularized area was not obvious, two to four optical fields with the highest MVD were photographed. However, only the most vascularized field was finally taken into account for further evaluation. Areas with a dense leukocytic infiltration were excluded from the photographic procedure because blood vessels were not obvious enough to be calculated.

Quantification on digital images was realized by using the SigmaScan V. 2.0 software (Jandel Scientific, Germany) on a Unisys CWP 5753 PC (Unisys Corp., San Jose, CA, USA). Further evaluation took place via the ColorEstimator V. 2.0, a specific application, developed in our Department in a Microsoft Visual Basic 5.0 (Microsoft Corp., Redmond, WA, USA) environment for the evaluation of histochemical and immunohistochemical color images.

Single endothelial cells or clusters of endothelial cells positive for FVIII were considered individual vessels. In each vessel the outline was identified and traced (Fig. 1b). The presence of blood cells or fibrin without any detectable endothelial cells was not sufficient to define a microvessel.

For each countable microvessel the following morphometric parameters were estimated: major axis length, minor axis length, area, perimeter and shape factor (4πXarea/perimeter^2). The variables entered into the statistical analysis were the mean values of the above five morphologic indices, the total count of microvessels per optical field (MVD), the total area occupied by them (TVA) and the number of vascular ramifications per 100-vessel sections (branching), as an expression of the complexity of the microvascular network. The whole procedure took place without any knowledge of the patients' clinicopathological data.

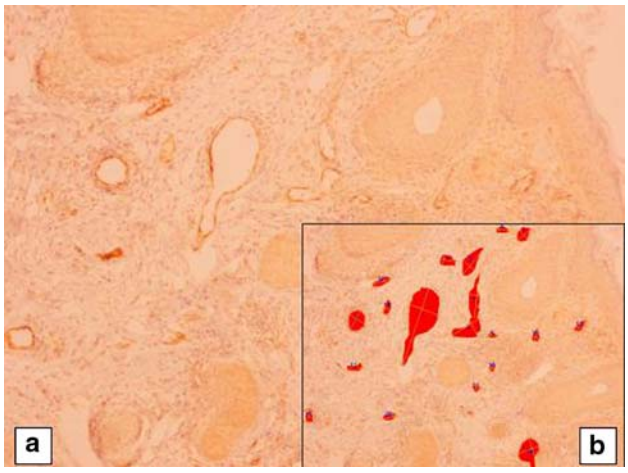

Fig. 1 **a** Hot spot area, with endothelial cells positive for FVIII, in a patient with Rosacea (X200). **b** The same as in **a** elaborated with image analysis software (×200)

Histological sections stained with Fast Blue RR method were also evaluated by image analysis for the quantification of mast cells in the dermis. The procedure of quantification concerning the measurement of these cells was identical with the procedure applied for the quantification of angiogenesis.

Statistical analysis

Initial statistics were based on frequency distribution tables and measures of central tendency and dispersity. Statistical inference was based on non-parametric statistics. Associations between categorical variables were assessed by chi-squared statistics with continuity correction whereas morphological variables were analyzed by Mann–Whitney *U*-test or Kruskal Wallis analysis of variance, as appropriate. Comparisons between pathological and control data were based on Wilcoxon signed ranks test. All tests were two-sided and level of statistical significance was set at 5%.

Results

Clinical findings

Of the 69 patients examined, 44 were females and 25 were males. The lesions predominated on the cheeks, nose, chin and forehead; they consisted of erythema (mild in 19 cases, moderate to severe in 50 cases), telangiectasias, papules (in 47 cases), pustules (in 30 cases) and rhinophyma in five patients. The erythematotelangiectatic rosacea (ETR) clinical type was present in 43 patients (62.3%) and the papulopustular type (PPR) only in 26 (37.7%). Dry appearance with scaling, often including the coexistence of seborrhoeic dermatitis, was present in 27.5% of the patients. Fifteen familial cases were reported. A history of migraine was given by 15 subjects (21.7%). Ocular manifestations were present in 26 cases (37.7%). Evidence of *H.pylori* infection was detected in 19 patients (26%). The mean duration of the disease was 4.1 years ± 3.9 SD.

Histological data

The most prominent and constant finding was solar elastosis, varying from severe to moderate in 54 cases and mild in the remaining 15. The second most prominent feature was grossly dilated, irregular vascular channels with oedema.

Demodex mites within hair follicles and sebaceous glands were present in 46 biopsy specimens (67%). Intense intrafollicular Demodex density was detected only in 24 cases (35%) whereas remnants of extrafollicular Demodex in 15 cases (21.7%).

The inflammatory infiltrate showed several histological patterns, being prominent in 17 cases (24.6%), moderate in 28 (40.5%) and mild in the rest (34.7%). Granuloma formation was found in 46 cases (67%).

In non-lesional skin biopsies, histological changes were aparrent but to a lesser degree than the clinically involved skin. Actinic elastosis, often severe, was common along with vascular dilatation. A mild inflammatory infiltrate was observed in all cases.

Immunohistochemistry and histochemistry data

The associations between variable clinicopathological and immunohistochemical parameters were evaluated statistically.

The PPR correlated significantly with older patients' age ($P = 0.020$), male sex ($P = 0.035$), high Demodex density ($P = 0.015$), ocular manifestations ($P = 0.003$) and high microvessel counts ($P = 0.002$).

The density of Demodex was found significantly higher in patients with PPR clinical type ($P = 0.015$), increasing also with patients' age ($P = 0.042$) but not with disease duration. Histologically high Demodex density was found to be related with the presence of giant cells ($P = 0.038$) and microabscesses ($P = 0.028$) in the dermis, although no correlation was detected between intrafollicular presence of Demodex and perifollicular inflammation.

The presence of *H.pylori* infection was unrelated to a specific clinical or histological pattern. Besides, no correlation was found between *H.pylori* presence and microvascular expansion or MCs dermal density.

The MVD, TVA and the morphometric parameters of microvessel shape and size except of the shape factor and branching had significantly lower values in the clinically uninvolved compared with the lesional skin (Table 1, Figs. 2, 3a, b).

High MVD and TVA values were found to correlate with the PPR clinical type ($P = 0.004$, 0.036) and the presence of ocular manifestations ($P = 0.002$, 0.032).

The MVD and TVA also, showed no correlation either clinically with patients' age or histologically with the degree of actinic elastosis or inflammation. However high MVD-values were found to correlate with granuloma formation in the dermis ($P = 0.056$, marginal significance).

The MCs number was found significantly higher in the lesional compared to clinically uninvolved skin ($P = 0.012$) (Figs. 4, 5a, b). Dermal MCs density and blood vessel number did not appear to have a direct correlation. On the contrary, statistically important results have been showed in the relation of MCs density with the duration of the disease ($P = 0.013$) (Fig. 6). There was no correlation found between MCs density and the type or intensity of the inflammatory infiltrate.

Table 1 Evaluated vascular morphometric parameters and MCs in lesional and non-lesional Rosacea skin

Test statistics[b]	*Z*	Asymp. Sing. (two-tailed)
MVD test-MVD control	−2.668[a]	0.008
MC test-MC control	−2.521[a]	0.012
Major AL test-major AL control	−2.310[a]	0.021
Minor AL test-minor AL control	−2.666[a]	0.008
Area test-area control	−2.310[a]	0.021
Perimeter test-perimeter control	−2.666[a]	0.008
SF test-SF control	−0.845[a]	0.398
TVA test-TVA control	−2.521[a]	0.012

[a] Based on negative ranks

[b] Wilcoxon Signed Ranks Test

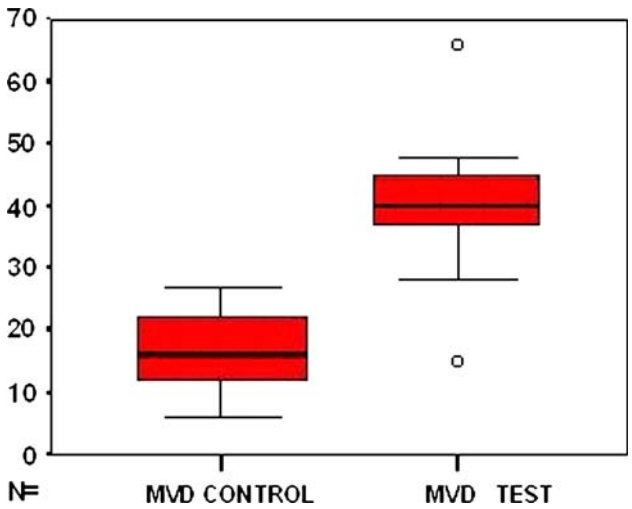

Fig. 2 Lesional skin presented with higher MVD compared with clinically uninvolved skin ($P = 0.08$)

Discussion

The importance of this study lies first in the comparison of the clinically involved and uninvolved skin of Rosacea patients with regard to angiogenesis and MCs number. Second, this study for the first time also correlated different clinicopathological findings of Rosacea with the two above-mentioned parameters, in an effort to shed some light in the pathogenesis of the disease. The sample of patients from which the uninvolved skin biopsy was taken was small (only 9), but one needs to consider the difficulty in convincing a patient to have a punch biopsy taken from seemingly non-diseased skin.

This investigation aimed to assess and evaluate the importance of the various factors that have been proposed to play a role in the pathogenesis of Rosacea. The most implicated factors are UVR-related damage [5, 21], angiogenesis [36] and Demodex mites [16, 17]. Moreover, this study assessed, for the first time to our knowledge, a possible role for MCs in the pathogenesis of this disease.

The histological findings from clinically involved skin showed indeed prominent solar elastosis, consistent with UV-mediated damage, (the extent of which could not be explained by age alone). The second most striking feature was expansion of the dermal microvasculature with grossly dilated, irregular vascular channels. Both these findings support the involvement of UVR-related damage and angiogenesis in Rosacea pathogenesis.

In our study, a statistically important correlation between MVD and TVA with PPR clinical type and ocular manifestations was found. This finding may suggest a close correlation of high vascular grade with the clinical severity of Rosacea. Moreover, statistically important results showed a relationship between MVD and granulomas formation in the dermis. This finding is in agreement with the theory that macrophages could influence various stages of angiogenesis. Macrophages produce a number of potent angiogenic cytokines and growth factors such as VEGF, TNFa, IL8 and bFGF. They also modulate events in the extracellular matrix through the secretion of extracellular matrix-degrading enzymes [4, 11, 24, 28]. Thus, pronounced angiogenesis seems to take place in more advanced stages of the disease.

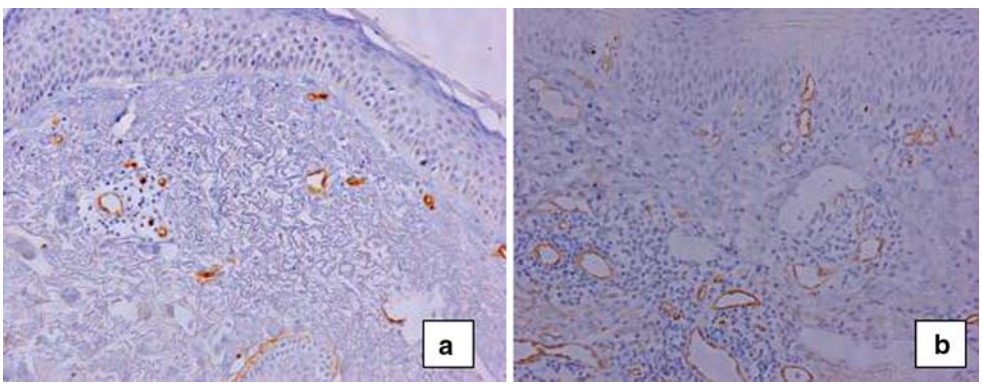

Fig. 3 Immunostaining against FVIII of microvasculature in lesional **(a)** and clinically uninvolved **(b)** skin in Rosacea. There is significant difference in extent of stained vasculature in the dermis between lesional and clinically non-lesional skin (×200)

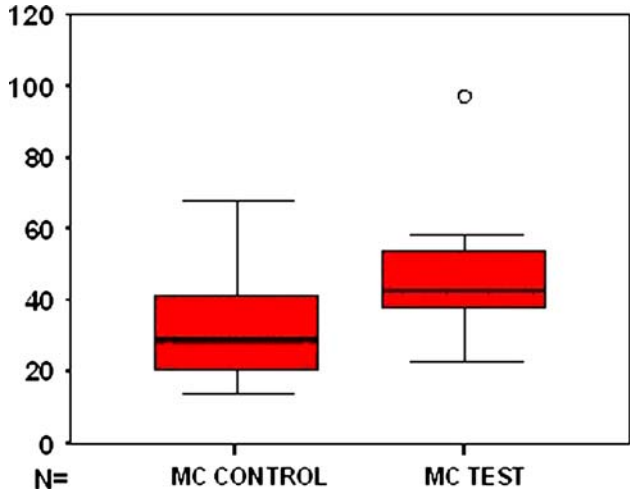

Fig. 4 Lesional skin presented with higher MCs numbers compared with clinically uninvolved skin ($P = 0.012$)

The study of clinically involved and uninvolved skin also gave some interesting results. First the involvement of angiogenesis in Rosacea pathology was again underlined by means of a significant difference of MVD and TVA between the two sample groups. Second, the MCs number was significantly greater in lesional compared to clinically non-lesional skin of Rosacea. This would imply a potential involvement for MCs in disease pathogenesis.

MCs are known to potentiate a number of inflammatory processes upon degranulation and to occur in increased numbers in conditions associated with angiogenesis [3, 20, 33]. There is much evidence that angiogenesis is related to MCs. Several MC mediators may act directly on endothelial cells to stimulate their migration and/or proliferation or may act indirectly by degrading connective tissue matrix to provide space for neovascular sprouts to form [25]. Angiogenic factors such as VEGF induce a direct chemotactic effect on MCs through enhanced adhesion to E and P selectin of postcapillary venules and also indirect through releasing from endothelial cells, MCs recruiting factors, such as stem cell factor and nerve growth factor [18, 19, 25].

However, in this study the number of MCs and blood vessels was not correlated. Moreover a strong correlation was found between MCs and the duration of the disease. These findings suggest that MCs may not play an essential early role in angiogenesis of Rosacea but rather may be related to the maintenance and maturation of blood vessels in Rosacea lesions, being necessary for the long-lasting functional integrity of the endothelium [22, 30]. Rhinophyma, a usually late manifestation of Rosacea is characterized by increased numbers of MCs [1, 32]. The above-mentioned findings support the significant role of MCs in the chronicity of Rosacea, as contributing agents to inflammation, angiogenesis and tissue fibrosis.

Some authors support that regressive changes are taking place in capillary walls in Rosacea, leading to irregularly shaped fused vessels and to loosened endothelial cells outside the vessels. The last being probably the source of neovascularization [27]. Recently an increased expression of VEGF in Rosacea, a major mitogen for dermal microvascular endothelial cells [31] is also reported.

It seems increasingly possible that Rosacea pathology is a multifactorial process, which opens up areas of research with regard to potential links between different contributing factors. Solar radiation may alter lymphatic and blood vessel function through the damage of the dermal support network of elastic and collagen fibers, resulting in the pooling of serum, inflammatory mediators and cellular degradation products. Proteases produced by inflammatory cells and angiogenic factors released in the context of chronic inflammation, worsen further the mechanical integrity of the upper dermis connective tissue, allowing the passive dilatation of the vasculature and increasing telangiectasia in Rosacea [13, 15, 26]. Recent studies have also shown that UVR stimulates angiogenesis, potentially bridging the divergent hypotheses of UVL exposure and vascular

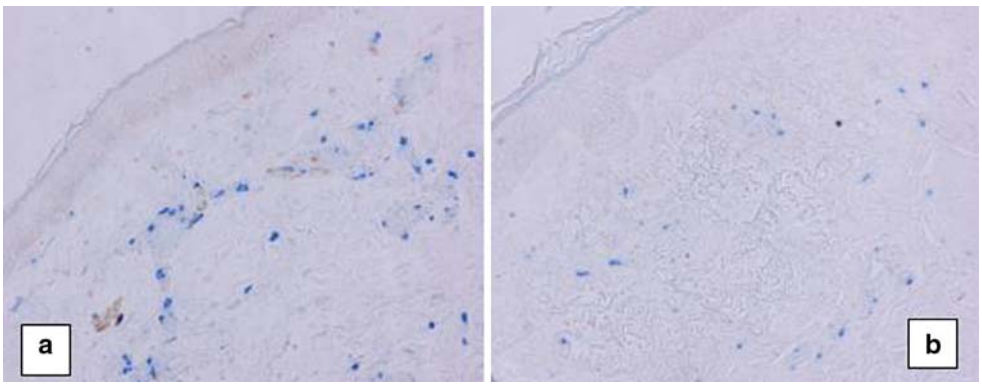

Fig. 5 An increase in the number of MCs is evident in the dermis of lesional skin **(a)** compared to clinically uninvolved skin **(b)** of a Rosacea patient (FBRR staining) (×200)

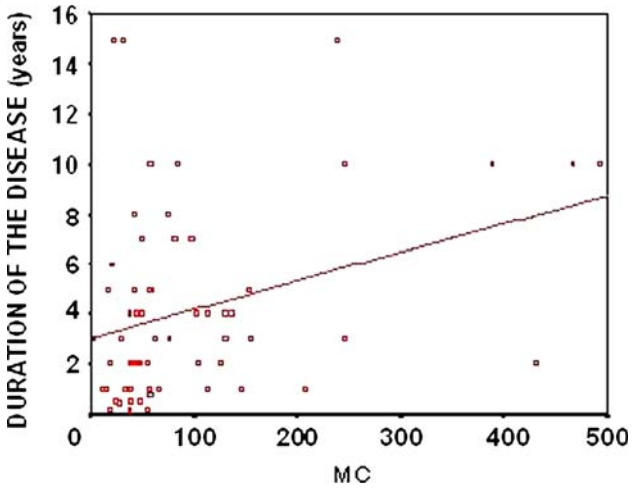

Fig. 6 Correlation of dermal MCs density with duration of the disease ($r = 0.298$, $P = 0.013$)

dysregulation on Rosacea pathogenesis [8, 23]. However, in our study the extent of solar elastosis did not seem to correlate with the extent of angiogenesis as measured by MVD and TVA.

Several authors have proposed that Demodex plays a pathogenic role in Rosacea [9, 16]. In our study, the density of Demodex was found to be significantly higher in the PPR clinical type of Rosacea. A heavy Demodex infestation probably increases the likelihood of follicular wall rupture and a consequent cell-mediated immune response [17]. Moreover, we found no correlation between perifollicular inflammation and the presence of Demodex intrafollicularly. It has been hypothesized that increased blood flow in dilated papillary dermal vessels of Rosacea provides a favorable environment for the multiplication of Demodex into the dermis [14]. However, we failed to reveal any relation between high Demodex density and MVD or TVA Besides, no correlation was found between *H.pylori* presence and microvascular expansion or MCs dermal density.

Based on our findings and taking into consideration the information from the literature, we conclude that solar degenerative elastosis, disorganized altered collagen, extrafollicular Demodex mites, mast cells and angiogenesis, all participate in various degrees and in different timing to the pathogenesis of Rosacea and elicit an inflammatory reaction in genetically predisposed persons.

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