SCIENTIFIC REPORT

Expression of vascular endothelial growth factor and its receptors in rosacea

Justine R Smith, Virginia B Lanier, Rita M Braziel, Katherine M Falkenhagen, Clifton White, James T Rosenbaum

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Background: Rosacea is a common chronic disease of unclear pathogenesis, characterised by inflammation and vascular abnormalities of the facial skin and ocular surface. Recognising that vascular endothelial growth factor (VEGF) is vasoactive and has inflammatory activities, the expression of this molecule and its receptors, VEGF-R1 and VEGF-R2, in rosacea was investigated.

Methods: Formalin-fixed, paraffin wax-embedded sections of skin obtained from 20 patients with rosacea were immunostained to detect expression of VEGF, VEGF-R1 and VEGF-R2, using an indirect methodology incorporating antigen retrieval. Adjacent sections were stained with haematoxylin and eosin.

Results: Biopsy specimens were characterised by perivascular and perifollicular lymphohistiocytic infiltration and dilated vascular channels. In addition to keratinocyte and epithelial staining, which was also noted in normal skin, vascular endothelium frequently stained positive for VEGF-R1 (14/20, 70%) and VEGF-R2 (20/20, 100%), but infrequently for VEGF (2/20, 10%). In most specimens, infiltrating leucocytes, including lymphocytes, macrophages and plasma cells, expressed VEGF (17/20, 85%), VEGF-R1 (20/20, 100%) and VEGF-R2 (20/20, 100%).

Conclusion: Expression of VEGF receptors, both by vascular endothelium and infiltrating mononuclear cells, is observed in rosacea. Although not expressed by endothelium, VEGF is present in epidermis and epithelium, and is expressed by infiltrating cells. VEGF receptor–ligand binding may contribute to the vascular changes and cellular infiltration that occurs in rosacea.

Rosacea is a chronic inflammatory condition of the central face, eyelids and ocular surface, which may cause facial disfigurement and vision-threatening keratoconjunctivitis despite therapeutic interventions.[1] Although there is an acknowledged lack of epidemiological data,[2] one frequently quoted study, involving a non-selected population of 809 office employees in four Swedish cities, estimates a prevalence of 10% in the general population.[3] The condition is more prevalent in adults, particularly in women and people of northern European ancestry.[1 2] Given the common occurrence of rosacea, a plethora of treatments have been promoted, yet recently, a rigorous review of treatment studies indicated that there was ''an urgent need for better quality, adequately designed randomized controlled trials'' on this topic.[4] An understanding of the basic mechanisms operating in rosacea is critical to that goal.

Although the literature contains hypotheses, the pathogenesis of rosacea remains unclear. Theories include: dysregulation of vascular diameter, perhaps exacerbated by hot beverages, alcohol and spicy food; a follicular-based immune response; heat-related or sun-related toxicity; and infection with microorganisms including *Demodex* mite strains or *Helicobacter pylori*.[2 5] Whatever the underlying aetiology, pathologists agree that rosacea is an inflammatory disease.[6] Microscopically, a mixed inflammatory infiltrate that comprises predominantly lymphocytes and macrophages is observed.[7] Vascular endothelial growth factor (VEGF) is expressed in normal epidermis and the epidermal appendages,[8] as well as in tissue obtained from patients with other inflammatory diseases that involve the skin and eyes.[9–12] Apart from participating in physiological angiogenesis and pathological neovascularisation, the molecule is vasoactive and has inflammatory activities.[13] In this study, we immunostained skin biopsy specimens from patients with rosacea to investigate the possibility that VEGF and its receptors, VEGF-R1 (flt-1) and VEGF-R2 (flk-1), might contribute to the pathogenesis of this disease.

MATERIALS AND METHODS

Tissue specimens

Altogether, 20 skin biopsy specimens were retrieved from the OHSU Pathology Archive (Portland, Oregon, USA) with the permission of the institutional review board. Patients included 13 women and 7 men, aged 31–82 years. Original dermatopathology reports were reviewed to verify the diagnosis of rosacea, and haematoxylin and eosin staining was performed to highlight tissue changes. At biopsy, specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Non-inflamed human skin, obtained during bariatric surgery, was processed in a similar manner.

Primary antibodies

Rabbit polyclonal anti-human VEGF and VEGF-R2 antibodies, and mouse monoclonal anti-human VEGF-R1 IgG_1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Working concentrations were 2.5 μg/ml for rabbit polyclonal antibodies and 1 μg/ml for the mouse monoclonal antibody. Rabbit IgG (Vector Laboratories, Burlingame, California, USA) and a mouse monoclonal IgG_1 antibody directed against the irrelevant antigen, KLH, (R&D Systems, Minneapolis, Minnesota, USA) were negative controls for these antibodies.

Immunohistochemical analysis

To detect VEGF and VEGF-R2, 3-μm thick, dewaxed and rehydrated tissue sections were boiled in a microwave for 10 min in 10 mM citrate buffer at pH 6. Sections were blocked for 1 h in TRIS-buffered saline, 0.1% bovine serum albumin, 0.3% Triton X-100 and 2% vol/vol normal goat serum. They were incubated overnight at 4℃ with primary antibody, diluted in blocking solution, and then incubated for 45 min at room temperature with biotinylated goat anti-rabbit antibody (Vector Laboratories), diluted 1:200 in blocking solution. Subsequently

Abbreviations: VEGF, vascular endothelial growth factor

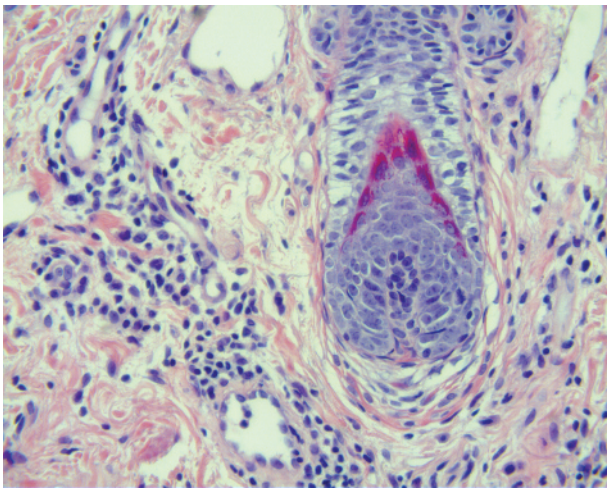

Figure 1 Typical perivascular and perifollicular mononuclear cell infiltrates and dilated vascular channels in the dermis of a patient with rosacea (haematoxylin and eosin: original magnification, ×400).

they were treated with a streptavidin–biotin complex conjugated to alkaline phosphatase (Vector Laboratories) at room temperature for 40 min. To visualise antibody complexes, sections were incubated with Fast Red (Biogenex, San Ramon, California, USA), according to the manufacturer's instructions. To detect VEGF-R1, an identical immunostaining procedure was followed; the blocking solution contained horse serum, and the secondary antibody was a biotinylated horse anti-mouse antibody (Vector Laboratories). Sections stained with irrelevant primary antibodies were included in every staining run.

RESULTS

Microscopical examination showed the characteristic finding of inflammatory cell infiltrates around vessels and hair follicles, in association with dilated vascular channels, in the dermis (fig 1). The infiltrate was heterogeneous, including a majority of lymphocytes and macrophages, but also plasma cells, multinucleated giant cells and neutrophils. In relatively severe cases, granulomas with multinucleated giant cells (n = 3) or microabscesses containing many neutrophils (n = 2) were noted. Degeneration of elastic fibres and collagen stroma was also observed.

In normal skin, the epidermis and the epithelium of eccrine and apocrine glands stained positively for VEGF, VEGFR-1 and VEGFR-2 (fig 2). VEGF-R1, but not VEGF or VEGF-R2, was also expressed by normal dermal vascular endothelium. As well as cytoplasmic staining, some nuclear uptake of the anti-VEGF-R1 antibody was noted.

Examination of skin biopsy specimens from patients with rosacea showed a similar positive staining of epidermis and glandular epithelium as was observed for normal skin. The dermal vascular endothelium frequently stained positively for VEGF-R1 (14/20 cases) and VEGF-R2 (20/20 cases), but infrequently for VEGF (2/20 cases). In most specimens, infiltrating leucocytes expressed VEGF (17/20 cases), VEGF-R1 (20/20 cases) and VEGF-R2 (20/20 cases) (fig 3A–C). However, while expression of VEGF, VEGF-R1 and VEGF-R2 could be localised to lymphocytes, macrophages and/or plasma cells, neutrophils seemed to express relatively little VEGF and to be receptor negative. Uptake of anti-VEGF and anti-VEGF-R1 antibodies by collagen and elastic fibres was considered to reflect tissue degeneration rather than specific antigen expression. Table 1 summarises the patterns of expression of VEGF, VEGF-R1 and VEGF-R2. Variable antigen preservation, an inherent problem when working with specimens obtained primarily for diagnostic purposes, prevented us from drawing conclusions about the relative intensity of staining.

DISCUSSION

Although the aetiology of rosacea remains poorly understood, inflammation plays a major role in the pathogenesis of the disease.[6] We identified VEGF and its receptors in facial skin biopsy specimens from 20 patients with rosacea. In addition to expression of VEGF, VEGF-R1 and VEGF-R2 by epithelium and epidermis, as was also observed in normal skin, lymphocytes and macrophages stained positively for these molecules in most specimens. Although VEGF was generally not expressed by the vascular endothelium, in most cases endothelial cells stained positively for its receptors. Because we studied specimens previously obtained for diagnosis, quantification of the relative

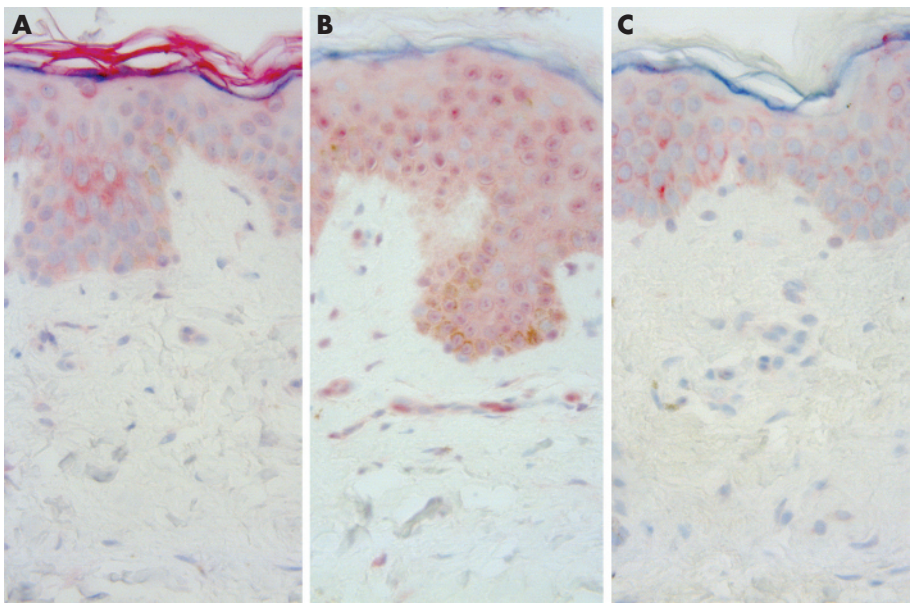

Figure 2 Photomicrographs of sections of normal skin that have been stained to detect (A) vascular endothelial growth factor (VEGF), (B) VEGF-R1 and (C) VEGF-R2. VEGF and both receptors are constitutively expressed by keratinocytes. Expression of VEGF-R1 by vascular endothelium within the dermis is also apparent (Fast Red with haematoxylin counterstain: original magnification, ×400).

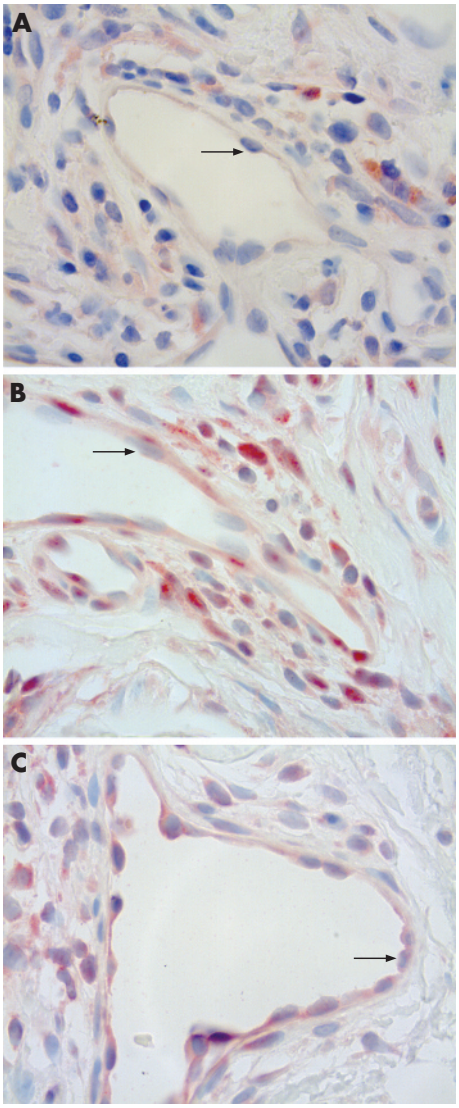

Figure 3(A–C) Photomicrographs of sections of facial skin from one individual with rosacea that have been stained to show the expression of (A) vascular endothelial growth factor (VEGF), (B) VEGF-R1 and (C) VEGF-R2. Infiltrating mononuclear cells stain positively for VEGF and both receptors, whereas the vascular endothelium (arrow) expresses VEGF receptors only (Fast Red with haematoxylin counterstain: original magnification, ×1000).

expression was not possible; research biopsies, consistently processed, with visualisation by confocal, as well as light, microscopy, could provide such information.

The demonstration of VEGF expression in normal skin epidermis and epithelium agrees with the findings of Viac *et al*,[10] who proposed that VEGF produced in this setting regulated vascular homoeostasis. In addition, we observed expression of VEGF-R1 and VEGF-R2 by epithelial cells. Normal dermal vascular endothelium was found not to express VEGF, as also reported by other researchers.[10 11] Descriptions regarding the expression of VEGF receptors by vascular endothelium in the dermis of healthy people are conflicting. One team reported complete absence of either receptor on endothelial cells,[12] whereas a second group noted expression of both receptors, particularly VEGF-R2.[11] In this study, we saw expression of VEGF-R2, but not VEGF-R1, in normal dermal vascular endothelium.

Table 1 Expression of VEGF, VEGF-R1 and VEGF-R2 in skin biopsy specimens from 20 patients with rosacea

	Epidermis positive total biopsy specimens*	Dermis positive total biopsy specimens*		
		Epithelium	Infiltrate	Vessels
VEGF	20/20 (100)	20/20 (100)	17/20 (85)	2/20 (10)
VEGF-R1	20/20 (100)	20/20 (100)	20/20 (100)	14/20 (70)
VEGF-R2	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)

VEGF, Vascular epithelial growth factor.
*Percentage of positive biopsy specimens

Consistent with our observations relating to rosacea, VEGF has been implicated in the pathogenesis of other chronic inflammatory diseases that involve the skin and eye. Detmar *et al*[11] reported overexpression of VEGF in the hyperplastic epidermis of skin biopsy specimens from patients with psoriasis. Subsequently, the same authors[14] showed that mice expressing the $VEGF_{164}$ splice variant under the control of a human keratin 14 promoter, leading to production of VEGF by basal keratinocytes, displayed a phenotype consistent with chronic skin inflammation. A later study showed that as adults, these mice spontaneously developed a skin condition that resembled human psoriasis and responded to treatment with the VEGF inhibitor, VEGF Trap.[15] Less extensive evidence has linked VEGF to systemic sclerosis,[9 10] bullous pemphigoid, dermatitis herpetiformis and erythema multiforme.[12]

Most of the literature on VEGF relates to its role in both physiological and pathological angiogenesis.[13] However, VEGF has chemoattractant activities that may be relevant to the pathogenesis of rosacea. Human monocytes migrate across collagen membranes and endothelial cell monolayers in response to VEGF,[16] and VEGF induces human T cell migration into skin allografts in the humanised SCID mouse.[17] In addition, VEGF promotes vascular dilatation and increases vascular permeability.[18 19] Inflammatory cell infiltrates and dilated vascular channels were typical in our patients. Keratinocytes and epithelial cells of all patients expressed abundant VEGF, and we observed expression of VEGF-R1 and VEGF-R2 by vascular endothelium in most specimens. Furthermore, VEGF and its receptors were expressed by infiltrating inflammatory cells. As reported by Xia *et al*,[15] production of cytokines by infiltrating cells would exacerbate any inflammatory process initiated by VEGF. Given the nature of our study, conclusions cannot be drawn regarding the point in disease progression at which VEGF might act, or regarding the relative importance of potential sources of VEGF.

The findings of this study indicate that the VEGF receptor–ligand binding may contribute to the vascular changes and cellular infiltration that occur in rosacea. Inhibition of VEGF is already being used therapeutically in the form of, for example, pegaptanib, an aptamer injected intravitreally in patients with neovascular age-related macular degeneration.[20] This method of treatment is under investigation for inflammatory diseases, including psoriasis.[21] Our results suggest the possibility that targeting VEGF receptor–ligand signalling might benefit patients with rosacea, particularly if hopes for surface delivery of anti-VEGF treatments[22] are realised.

Authors' affiliations
Justine R Smith, Virginia B Lanier, Katherine M Falkenhagen, James T Rosenbaum, Casey Eye Institute, Oregon Health and Science University, Portland, Oregon, USA
Rita M Braziel, Department of Surigical Pathology, Oregon Health and Science University, Portland, Oregon, USA

Clifton White, Department of Dermatology, Oregon Health and Science University, Portland, Oregon, USA
James T Rosenbaum, Department of Medicine, Oregon Health and Science University; Department of Surgical Pathology, Oregon Health and Science University, Portland, Oregon, USA

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Correspondence to: Dr J R Smith, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Biomedical Research Building, Mail Code L467AD, Portland, OR 97239, USA; smithjus@ohsu.edu

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