Increased Expression of Vascular Endothelial Growth Factor in Pyogenic Granulomas

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The expression of vascular endothelial growth factor (VEGF) was analysed in biopsy samples from patients with pyogenic granuloma. The results disclosed the presence of a strong VEGF signal in pyogenic granulomas, which are constituted by a vast majority of cells of endothelial lineage. A marked positivity was evident in areas of proliferating endothelial cells without vessel lumen formation. In the same respect, staining for VEGF was less marked in the vessels with a well-developed lumen. The fact that VEGF production appears to be limited to endothelial cell precursors or immature endothelial cells prior to the complete development of the vessels, leads to the possibility that VEGF may act as an autocrine factor in circumstances of endothelial cell stimulation. *Key words: VEGF; pyogenic granuloma; endothelial cells.*

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Pyogenic granuloma is a common, acquired disease, involving vascular proliferation in the skin and mucous membranes. The main histopathological features of pyogenic granuloma include active endothelial cell proliferation, resulting in lobules of capillaries separated by septa of connective tissue. In spite of its frequency, the pathogenetic mechanisms leading to the development of pyogenic granuloma are still unknown. The angiogenic potential of pyogenic granuloma tissue has been highlighted recently by Ribatti et al. (1), who have demonstrated that pyogenic granuloma may promote angiogenesis by releasing angiogenic factors. These authors, however, did not identify the factors involved in the proliferative phenomenon. Accordingly, the possibility that angiogenic factors are involved in the pathogenesis of pyogenic granuloma deserves specific attention. Vascular endothelial growth factor (VEGF), a strong stimulator of endothelial cell proliferation, is a putative candidate as an angiogenic mediator in pyogenic granuloma. VEGF is usually generated by non-endothelial cellular types, e.g. vascular smooth muscle cells or tumoral cells (2). In basal conditions, endothelial cells do not produce VEGF; however, a strong VEGF expression can be obtained by submitting endothelial cells to hypoxia or oxidative stress (2–4).

The aim of this study was to investigate the expression of VEGF in tissue samples obtained from patients affected by pyogenic granuloma.

MATERIALS AND METHODS

Biopsy specimen

Five pyogenic granulomas excised from 5 patients were fixed in 10% formalin and embedded in paraffin according to routine procedures. The biopsies corresponded to 2 males and 3 females, aged 16–60 years (mean $\pm$ SD 35.2 $\pm$ 17.6 years). At the time of biopsy, lesions of pyogenic granuloma dated from 3 to 12 months (mean $\pm$ SD 7.8 $\pm$ 3.9 months) in the different patients. Samples from normal skin obtained from 2 healthy volunteers were used for comparative purposes.

Peptide synthesis, coupling to carrier and immunizations

The peptide sequence APMAEGGGQNHHEVVKFM, which spans residues 1–18 of the 4 main isoforms of VEGF (2), was synthesized by f-moc chemistry. After cleavage from the resin, the peptide was purified by HPLC and quantitated by amino acid analysis. For coupling to a carrier, 2 mg of keyhole limpet haemocyanin (KLH) was dissolved in 1 ml PBS and mixed with 6×10^3-fold molar excess of sulfo-SMCC (Pierce) for 1 h at 30°C. The reaction product was then passed through Sephadex G-25 equilibrated with 100 mM sodium phosphate buffer (pH 8) to remove free sulfo-SMCC. The purified KLH-sulfo-SMCC was then reacted with 2 mg of peptide overnight, at room temperature, in the dark while being stirred. The reaction mixture was then dialysed against PBS. The amount of attached peptide in the conjugate, as determined by amino acid analysis, was approximately 3000 mole peptide/mole KLH.

Polyclonal anti-VEGF antibodies were raised after immunizing New Zealand rabbits with multisite intradermal injections of 500 µg of KLH-peptide emulsified in complete Freund's adjuvant with monthly booster injections. The animals were bled at monthly intervals, starting 2 weeks after the second injection. The specificity and sensitivity of the antibody were tested by ELISA against a synthetic VEGF (165 amino acids isoform, Sigma, Madrid, Spain), and by immunocytochemistry on stimulated vascular smooth muscle cells. The anti-VEGF antibody used was of similar efficiency to a commercial antibody, (Sigma, St Louis, MO, USA), as demonstrated in VEGF-blockade studies in our laboratory (5).

Immunohistochemistry

Ten HE-stained sections of each specimen were compared. Three sections were mounted on each slide for immunohistochemical study of each antibody. Paraffin blocks were sectioned at 3–5 µm and attached to poly-L-Lysine-coated glass slides. The paraffin sections were deparaffined by xylene and ethanol gradient, incubated in trypsin for 10 min, and then in 3% H_2O_2 for 30 min. After washing in PBS, sections were incubated with 10% swine serum (DAKO) for 20 min, to block non-specific binding. All these procedures were carried out at room temperature. Primary antibodies were applied overnight at 4°C and then 60 min at room temperature. After washing with PBS, the slides were incubated with biotin-labelled swine anti-rabbit antibodies (DAKO) for 40 min, washed with PBS and then incubated with streptavidine-peroxidase (DAKO) for 40 min.

After washing, the immunoreactions were visualized by incubation with the substrate diaminobenzidine (DAKO), the reaction being stopped with PBS and the slides being counterstained with haematoxylin and mounted in oil mounting media.

Samples treated with pre-immune sera obtained from the same rabbits prior to starting the immunization protocol were used as controls. Conventional histology techniques, as well as routine immunohistochemistry with anti FVIII, CD31, CD34 and actin were performed on the same samples. Also, as aforementioned, normal skin was used as a negative control.

RESULTS

All the samples included in this study had histopathological features of pyogenic granuloma in different stages of evolution. Briefly, the most recent lesions consisted of a granulation tissue proliferation composed of numerous capillaries and venules disposed radially to the skin surface, which was often eroded and covered with crust.

The stroma was oedematous and contained a moderate inflammatory infiltrate composed of a mixture of lymphocytes and neutrophils. Fully developed lesions of pyogenic granuloma exhibited a polypoid shape and consisted of several lobules of well-developed capillaries and venules with plump endothelial cells separated by septa of connective tissue of variable thickness. These late lesions showed a lesser degree of inflammatory infiltrate; often a collarette of adnexal epithelium partially embraced the lesions at their periphery. Samples from a representative patient are shown in Figs. 1A – F. Figs. 1A and C correspond to negative staining with pre-immune serum at different degrees of magnification. Many of the cells in the pyogenic granuloma expressed the FVIII-related antigen (Fig. 1E), CD31 and CD34, which is a marker of activated endothelial cells in adults (6). Some zones with FVIII positivity consisted of solid areas with no vessel lumen formation, whereas other areas exhibited the appearance of well-developed vessels surrounded in some cases by actin positive cells, corresponding to pericytes or vascular smooth muscle cells (Fig. 1F). Images of CD31 and CD34 were basically similar to those of FVIII, albeit with a more intense staining of the cells outlining the capillary walls.

A definite and similar pattern of positivity for the anti-VEGF antibody was detected in all the samples we tested. As shown in Figs. 1B and D, the strongest positivity was detected in areas constituted by a proliferation of endothelial cells within the oedematous stroma, but with no capillary lumen formation. A significant background staining was also detected in intercellular areas, corresponding to matrix staining. Positivity for VEGF was less marked in vessels with well-formed lumina (Fig. 1D), which showed a clear-cut actin positivity (Fig. 1F). Furthermore, normal skin resulted negative for VEGF staining with the exception of some staining with the anti-VEGF antibody, which was detected in avascular areas of the corneal strate in normal skin and corneal strate covering the pyogenic granulomatous lesions. These images are not shown because they are identical to previous reports in the literature (2, 7, 8). All the images shown in Figs. 1A – F belong to consecutive samples from the same preparation.

DISCUSSION

We have investigated the expression of VEGF in pyogenic granulomas in order to ascertain the putative role of this factor in the development of the disease. The present results suggest that VEGF is definitely involved in the pathogenesis of pyogenic granuloma. The expression of VEGF on pyogenic granuloma can be related to that observed during wound healing (7). In this regard, pyogenic granuloma may be considered as an exuberant expression of granulation tissue formation in prolonged healing processes. An increase in VEGF expression has also been described in other cutaneous entities (8), e.g. vesiculo-bullous diseases, but, as reported (2, 7, 8) and as we found in the present study, VEGF expression is absent in normal skin. The present findings have potential pathophysiological implications, namely, the possibility that endothelial cell precursors express VEGF *in vivo*. Similar endothelial positivity for VEGF has been recently reported by Takano et al. (9) in brain glioblastomas, forming microvessel "hot spots". The background positivity for VEGF found in the present study is coincident with that reported by other authors (10) and can be traced to the binding of VEGF to mucopolysaccharides and heparinoid molecules in the intercellular tissue. Similarly, some degree of positivity in the keratinocyte layer is compatible with recent evidences showing that VEGF synthesis can be elicited in cultured keratinocytes treated with inflammatory cytokines (11). Moreover, antisense oligonucleotides can inhibit VEGF expression in human epidermal keratinocytes in culture (12).

In several *in vitro* studies, VEGF gene expression was induced on endothelial cells by hypoxia, chemical manoeuvres resulting in hypoxia and oxidative stress. However, to date no reports exist on the expression of VEGF in endothelial cells in tissues obtained from adult individuals. The present findings open the possibility that in certain circumstances VEGF may act as an autocrine agent for endothelial cell proliferation. Recent observations by Nomura et al. (3) provide support for this possibility; these authors have found that antisense oligodeoxyribonucleotides against VEGF mRNA inhibit proliferation of endothelial cells in culture. Studies from Wizigmann-Voos et al. (13) have described that VEGF mRNA is present in stromal but not endothelial cells of capillary haemangioblastomas of patients with von Hippel-Lindau disease. VEGF protein, however, was detected in perivascular areas, suggesting a paracrine accumulation. These findings differ from ours in pyogenic granulomas and provide further support for the existence of different pathogenetic sequences involving VEGF in either haemangioblastomas or pyogenic granulomas. Accordingly, in some vascular neoplasms VEGF is produced by a source outside the vessel wall and acts on target endothelial cells. On the other hand, the fact that endothelial cell precursors might be the VEGF source in pyogenic granuloma, would determine that VEGF production decreases at the time of the mature vessel formation. At present, no data are available to identify the mechanisms triggering VEGF production in pyogenic granuloma. Results from our laboratory (Alvarez Arroyo et al., unpublished data), have shown that locally increased concentrations of reactive oxygen species strongly stimulate VEGF production by endothelial cells. Therefore, the existence of local inflammatory cytokines might explain the induction of VEGF (11). Moreover, recent data from our laboratory have related the appearance of intense VEGF mRNA and protein expression with disruption of inter-endothelial adherens junctions (5).

The finding that VEGF positivity appears to be less pronounced in vessels with well-developed lumina may be relevant in the natural history of pyogenic granuloma. In fact,
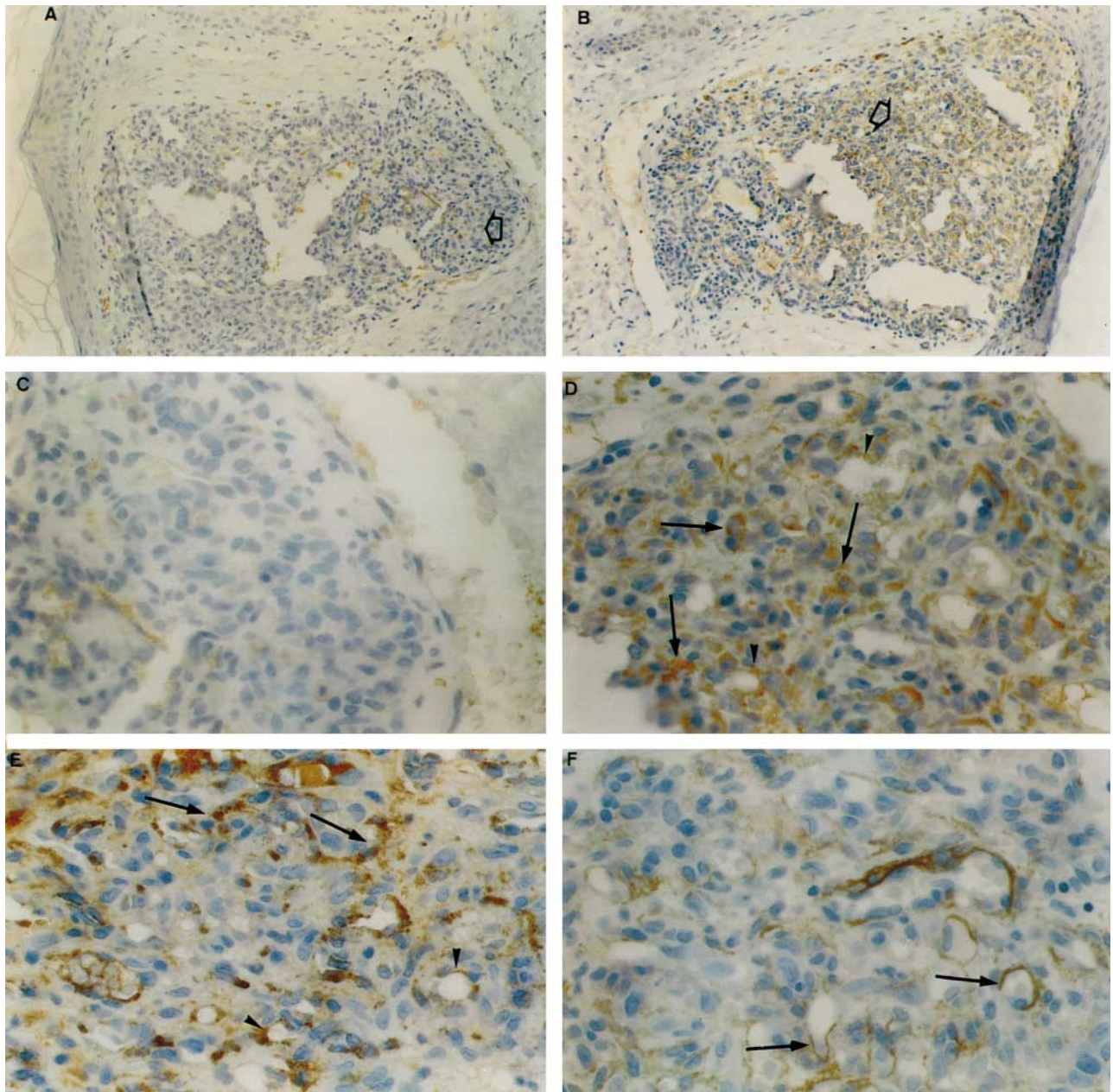
Fig. 1. (A) Sample stained with rabbit pre-immune serum. No VEGF signal is detected (×40). Empty arrow indicates the area magnified in the following figures. Nuclei correspond to endothelial cells. (B) Consecutive section of the same preparation shown in (A) (×40), showing intense positivity with the anti VEGF antibody. Empty arrow indicates the area magnified in the following figures. (C) Detail (100× magnification) of one field from the pre-immune control of (A). No VEGF staining is evident. (D) Detail (100× magnification) of 1 field from (B), showing intense VEGF staining. Mostly, positivity for VEGF is evident in cells with no vessel lumen formation (*black arrows*). VEGF positivity can be seen, with less intensity, in cells surrounding some of the vessels (*arrowheads*) and in intercellular matrix areas. (E) Factor VIII-related antigen staining in samples consecutive to those of (C) and (D) (100×). FVIII positivity was evident both in cells surrounding the vascular lumen (*arrowheads*) as well as in packed groups of cells showing no lumen (*black arrows*). The distribution of FVIII staining is similar to that of VEGF shown in (D), except that matrix staining was absent with FVIII. (F) Alpha smooth muscle actin (ASMA) positivity in the muscular layer of the walls of blood vesels (*arrows*). Endothelial cells lining lumina and interstitial endothelial cells are ASMA negative (×100). The sample corresponds to preparations consecutive to those in the other parts of this figure.

this phenomenon may help to clarify the autolimited nature of pyogenic granuloma lesions. In this regard, the hypothesis can be raised of the existence of a sequence including early VEGF expression by endothelial cells precursors and subsequent endothelial cell proliferation; once the vessels are developed enough to constitute a complete lumen and, therefore, permit the circulation of blood, the subsequent increase in oxygen concentration favours the inhibition of VEGF expression. This phenomenon might lead to subsequent involution of capillary proliferation and ultimately to the healing of the pyogenic granuloma. Takahashi et al. (14) have described VEGF positivity in the cytoplasm of both endothelial cells and pericytes of microvessels in the proliferating phase of haemangiomas; this positivity disappeared in the involuting

phase of these neoplasms. A major dependence from locally formed VEGF would explain the autoinvolutive nature of pyogenic granulomas. Furthermore, the possibility exists that either the application of local anti-VEGF treatments or increasing the local oxygen concentration, both manoeuvres which can switch-off the VEGF expression, are potentially useful therapeutic alternatives in severe or recurrent cases of pyogenic granuloma.

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