OVEREXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS ENDOTHELIAL CELL RECEPTOR KDR IN TYPE 1 LEPROSY REACTION

PAOLO FIALLO, ANDREA CLAPASSON, ANNA FAVRE, AND CARLO PESCE
Centro Nazionale di Riferimento per la Malattia di Hansen e la Dermatologia Tropicale (CIRLEP), Dipartimento di Scienze della Salute, Università di Genova, Genova, Italy;
Centro di Anatomia Patologica, DISTBIMO, Università di Genova, Genova, Italy

Abstract. The sites of expression of vascular endothelial growth factor (VEGF) and of KDR, its endothelial cell receptor, were investigated in leprosy reaction Type 1, or reversal reaction (RR), by immunohistochemistry and in situ hybridization. In comparison with nonreactional leprosy, overexpression of both VEGF and KDR was seen in granuloma cells, especially epithelioid and foreign body–type giant cells, the epithelium and the vascular endothelium of RR specimens. In granuloma cells, hybridization for VEGF was stronger than immunostaining, a finding that may reflect the rapid turnover of VEGF in an immunologically dynamic situation such as RR. In the epidermis, double immunohistochemistry revealed VEGF overexpression in CD1a-positive dendritic cells. The VEGF may not only be relevant for hyperpermeability and mononuclear cell differentiation (the key morphologic features in the acute, clinically evident phase of RR), but it could also be implicated in RR onset, when dendritic cells are activated in response to antigen stimulation.

INTRODUCTION

A reaction is an acute inflammatory episode superimposed on the usually uneventful course of leprosy. There are 2 types of leprosy reaction, Type 1, or reversal reaction (RR), which occurs mainly in borderline leprosy, and Type 2, or erythema nodosum leprosum, which develops in lepromatous leprosy and occasionally in mild forms of borderline leprosy. The histological hallmarks of RR are conspicuous dermal edema, often associated with dispersion of the granuloma, the appearance of foreign body–type giant cells, and an increased number of lymphocytes.

Reversal reaction, a delayed hypersensitivity response against Mycobacterium leprae antigens, is characterized by a T helper (Th)-1–like response phenotype in the peripheral blood mononuclear cells in the presence of interferon gamma (IFN-γ). Interleukin (IL)-4 is absent, but IL-12 is consistently expressed. The IFN-γ–producing CD4+ cells and T cytotoxic cells are selectively increased with clearing of bacilli and concomitant tissue damage. In tissue samples, a similar pattern of IFN-γ expression and absence of IL-4 has also been demonstrated in RR by reverse transcriptase–polymerase chain reaction.

Vascular endothelial growth factor (VEGF), a cytokine originally described as a tumor cell product under the name of vascular permeability factor, is expressed in normal adult tissues, including the epidermis. VEGF enhances permeability of normal venules and small veins and is a selective mitogen for endothelial cells. In the pathologic setting, VEGF regulates angiogenesis, enhances migration and activation of macrophages in granulomatous inflammation, and is overexpressed in delayed hypersensitivity skin reactions.

This study investigated the sites of expression of VEGF and of KDR, its endothelial cell receptor, in RR in comparison with nonreactional leprosy across the spectrum of the disease.

PATIENTS AND METHODS

Tissue samples and histology. Skin biopsies from 7 patients with RR (6 borderline tuberculoid and 1 borderline lepromatous) were retrieved from the histologic files of the National Reference Center for Leprosy of Italy. The Ridley-Jopling leprosy classification was applied to define the leprosy diagnosis across the spectrum of the disease. Additional biopsies of leprosy without histologic signs of RR (7 borderline tuberculoid, 3 borderline lepromatous, and 4 lepromatous leprosy) were retrieved. These 21 biopsies were subsequent entries in the registry.

The specimens were fixed in 10% formaldehyde for 24 hr. Sections were stained with hematoxylin and eosin for general microscopic examination and a modified Fite-Faraco procedure for M. leprae. RR was identified histologically by conspicuous edema with dispersion of the granuloma and foreign body–type giant cells. Specimens of skin angioma (n = 4) and normal skin (n = 3) were used as controls.

In situ hybridization and immunochemistry. In situ hybridization (ISH) and immunohistochemistry were performed on 5-μm sections of paraffin-embedded tissue mounted onto slides coated with polylysine. For microwave antigen retrieval, sections were placed in Coplin jars with a preheated 10 mM sodium citrate buffer (pH 6) and heated in a household microwave oven operating at a frequency of 2.45 GHz. The samples for ISH were microwaved 3 times at 800 W and twice at 600 W for 3 min; those for immunohistochemistry were microwaved first at 800 W (once) and later at 600 W (4 times). Every 3 min, the fluid level was controlled and adjusted, if necessary. The slides were cooled in distilled water and transferred to phosphate-buffered saline (PBS).

For ISH, sections were placed at room temperature in a humid chamber covered with paper absorbed with 4× standard saline citrate (SSC), 50% (v/v) deionized formamide, where they were equilibrated in prehybridization solution (4× SSC, 50% v/v deionized formamide, 1× Denhardt medium, 5% w/v dextran sulfate, 500 μg/mL salmon sperm DNA, and 250 μg/mL tRNA [Escherichia coli]) for 1 hr. Slides were then hybridized with 100 μL of the same solution containing ~12 ng of DNA probe. The oligonucleotide sequence used to generate the antisense DNA probe (49 mer) was 5′-CGC ATC GCA TCA GGG GCA CAC AGG ATG GCT TGA AGA TGT ACT CGA TCT C-3′. This sequence corresponded to human VEGF bases 258–306. The probe

Copyright © 2002 by The American Society of Tropical Medicine and Hygiene
was labeled with digoxigenin-uridine-tri-phosphate by the 3’ tailing method (Roche Diagnostics, Milano, Italy).

After overnight hybridization at 37°C, the sections were washed twice in 2× SSC (1 hr at room temperature), twice in 1× SSC (1 hr at room temperature), once in 0.5× SSC (30 sec at 37°C), and once in 0.5× SSC (30 sec at room temperature). Sections were then washed for 10 min in Buffer 1 (100 mM Tris-HCl, pH 7.5; 62.5 mM NaCl) and blocked by incubation for 30 min after addition of 2% (v/v) normal goat serum. The sections were later incubated in Buffer 1 with 1% normal goat serum and alkaline phosphatase–conjugated anti-digoxigenin antibody (1:500; Roche) for 2 hr, and washed twice (15 min) in Buffer 1 and twice (2 min) in Buffer 2 (100 mM Tris-HCl, pH 9.5; 62.5 mM NaCl; 50 mM MgCl₂). Sections were immersed in Buffer 2 containing nitroblue tetrazolium as chromogen and 5-bromo-4-chloro-3-indolylphosphate as substrate (1:100 stock solution), 1 mM tetramisole, in the dark for 3–5 hr. Sections were then washed for 10 min in Buffer 1 with 1% normal goat serum and alkaline phosphatase–conjugated anti-digoxigenin antibody (1:500; Roche) for 30 sec at 37°C, and incubated with the antibody at 4°C.

Sections for double immunostaining were incubated with lepromatous leprosy) and moderately in 11 additional controls.

Sections for double immunostaining were incubated with 0.3% H₂O₂ solution in PBS to block endogenous peroxidase, washed in PBS, and incubated overnight with the first antibody (CD1a) at 4°C. The blue alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) technique was followed for antibody detection with naphtol AS-BI phosphate (Sigma N2125), Fast Red TR salt (Sigma) and 1 mM tetramisole (Sigma) as substrate solution for 45 min at room temperature. Sections were counterstained with Mayer’s hematoxylin and mounted in aqueous mounting medium. Incubation without primary antibodies was used as negative controls.

Sections for double immunostaining were washed twice in 2× SSC, and incubated for 3 hr. A mouse anti-rabbit antibody was applied for 30 min and the envision dextran-peroxidase complex added for a further 30 min of incubation. The peroxidase reaction was developed with aminoethylcarbazole. Sections were counterstained with methyl green and mounted in gelatin.

The following antibodies were used for immunohistochemistry: (1) anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:40 (starting at 200 μg/mL) (1:100 for double immunostaining) in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 1% bovine serum albumin; (2) anti-KDR2 monoclonal antibody (Sigma) at a dilution of 1:25 in PBS–1% bovine serum albumin; and (3) CD1a goat polyclonal (Santa Cruz Biotechnology) at a dilution of 1:100.

RESULTS

Epidermal cells hybridized strongly for VEGF in 9 specimens (all 7 RR specimens and 2 nonreactional specimens with lepromatous leprosy) and moderately in 11 additional specimens of nonreactional leprosy (Table 1). In one patient with lepromatous leprosy, samples of the epidermis did not label for VEGF. Cells of the spinous layer labeled most intensely, especially in relation to large subepidermal areas of inflammatory infiltrate. The portions of epidermal lining more distant from the granuloma showed mild ISH labeling, as did normal control specimens.

Only in specimens of RR was epidermal labeling for VEGF associated with intense positivity in the granuloma (Figure 1). The ISH showed VEGF to be localized in activated macrophages, epithelioid, and foreign body–type giant cells (Figure 2). This pattern was in sharp contrast to that of the surrounding lymphocytes, which were invariably negative for VEGF. The ISH positivity in the granuloma of non-reactional leprosy was mild. In RR specimens, additional dermal structures overexpressing VEGF were vessels, especially capillaries, and eccrine glands. Nerves occasionally expressed VEGF, but at lower levels of intensity. No staining was seen via hybridization with a sense-oriented probe for VEGF as a negative control. Angioma endothelial cells labeled strongly for VEGF.

The VEGF immunochemistry showed a staining distribution similar to ISH, but positive cells were fewer in the epithelium and the granuloma (Figure 3A). Immunohistochemistry for KDR revealed a distribution of positivity similar to that of VEGF ISH, with vascular endothelium staining being particularly strong (Figure 3B).

In RR specimens, double staining with CD1a and VEGF revealed abundant CD1a-positive cells, many of which had disrupted cytoplasm, that also stained for VEGF (Figure 4C). In nonreactional leprosy specimens, double staining showed a reduced number of CD1a-positive cells with occasional VEGF double staining.

### Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Classification</th>
<th>Epidermis</th>
<th>Granuloma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>BT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>BT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>BT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>BT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>BT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>BT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>BT-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>BT-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>BT-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>BT-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>BT-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>BT-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>BL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>BL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>BL</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>17</td>
<td>BL-RR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>LL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>LL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>LL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>LL</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* BL = borderline lepromatous; BT = borderline tuberculoid; LL = lepromatous; NA = not applicable; RR = reversal reaction. The difference between reactive and nonreactive epidermis is highly significant (P = 0.002); the difference between reactive and nonreactive granuloma cells is highly significant (P = 0.0004; Mann-Whitney 2-sample nonparametric test).

In RR specimens, double staining with CD1a and VEGF revealed abundant CD1a-positive cells, many of which had disrupted cytoplasm, that also stained for VEGF (Figure 4C). In nonreactional leprosy specimens, double staining showed a reduced number of CD1a-positive cells with occasional VEGF double staining.
Figure 1. *In situ* hybridization (ISH) for vascular endothelial growth factor. There is strong vascular endothelial growth factor hybridization of epidermal and granuloma cells, including macrophages and epithelioid cells in reversal reaction (left). Hybridization is weaker in the epidermis of a case of nonreactive borderline tuberculoid leprosy, where only few granuloma cells are positive (right) (ISH, ×250).

Figure 2. Close-up of the granuloma in reversal reaction discloses vascular endothelial growth factor in epithelioid and giant cells. Conspicuous edema is associated with granuloma cell dispersion (*in situ* hybridization, ×400).
Figure 3. (A) Immunohistochemistry for vascular endothelial growth factor shows moderate positivity in the cytoplasm of macrophages, epithelioid cells, and 2 multinucleated giant cells (vascular endothelial growth factor [VEGF] immunostaining, ×400). (B) Endothelia are strongly positive for endothelial cell receptor KDR immunohistochemistry (×800). (C) Double immunostaining shows numerous dendritic cells that are also positive for VEGF (×400).
DISCUSSION

This study on the sites of expression of VEGF and its receptor KDR in RR in comparison with nonreactional leprosy was prompted by several observations. The VEGF is overexpressed in 2 well-characterized models of delayed hypersensitivity, allergic contact dermatitis, and tuberculoid reaction; is an important regulator of angiogenesis in chronic granulomatous inflammation; increases permeability of normal venules and small veins; and enhances activation and migration of monocytes. A corollary consideration was that the typical histologic features of RR—edema and foreign body-type giant cells superimposed on the microscopic pattern of a borderline form of leprosy—could be the result of these functional changes. In our experience, VEGF and KDR were overexpressed in RR in comparison with nonreactional leprosy in the granuloma cells, vascular endothelium, and overlying epidermis.

Within the granuloma, VEGF ISH showed a distribution in the cells of mononuclear-macrophage lineage, especially epithelioid cells, similar to that of VEGF immunohistochemistry. The ISH, however, was more intense and labeled more cells, a finding that may reflect the rapid turnover of VEGF protein in this immunologically dynamic situation, when a new wave of activated macrophages join the preexisting granuloma. Overexpression of KDR in cells of the mononuclear lineage suggests that these cells could activate themselves to produce VEGF in an autocrine fashion.

The VEGF was expressed in the foreign body-type giant cells of RR. The appearance of this giant-cell type is one of the histologic hallmarks of RR. In nonreactional leprosy, apart from the relatively small multinucleated cells with ample cytoplasm corresponding to bacilli-laden phagocytes of lepromatous leprosy, giant cells are of Langhans type. This giant-cell type is occasionally found admixed with epithelioid cells in borderline tuberculoid and tuberculoid forms. Giant-cell type is occasionally found admixed with epithelioid cells in borderline tuberculoid and tuberculoid forms. This occurrence of foreign body-type giant cells was achieved with IFN-γ and IL-3. These data are at variance with the occurrence of foreign body-type giant cells in RR because IL-4 is absent from this condition. We postulate that cytokines other than those considered by McNally and Anderson may regulate in vivo differentiation of giant cells to the foreign body-type pattern.

Although this retrospective study was not designed to investigate differences in VEGF expression in the subsequent stages of RR through serial biopsies, our results on vessels and epidermis indicate that this mediator could play a role throughout the natural history of RR. On the one hand, VEGF and KDR overexpression in endothelial cells of RR indicate that VEGF could be implicated in the vascular changes, in particular tissue edema, that characterize RR at early and later stages of development. Edema, which is minimal in nonreactional leprosy, appears in extracellular location within and outside the granuloma in early RR and becomes profuse in its acute, clinically apparent stage, when breakdown and dispersal of the granuloma is seen.

On the other hand, VEGF could be involved in the very onset of RR. Several observations indicate that RR is triggered by Langerhans cell activation. In fact, OKT6-positive cells increase in number, aggregate in clusters, and show occasional disintegration at RR onset. Furthermore, in RR, the 3A8 epitope of mycobacterial 30-kDa proteins is more strongly expressed on CD1a dendritic Langerhans cells both in the epidermis and the dermis. Although in our experience ISH showed extensive VEGF production in the epidermis overlying RR granulomas, double immunostaining with CD1a showed VEGF overexpression of epidermal dendritic cells in RR. This observation suggests that dendritic cells could constitute storage sites of VEGF and that discharge of this mediator could be relevant for signal transmission during the initial phase of RR.

Financial support: This work was financed by Associazione Italiana Amici di Raoul Follereau, Bologna, Italy.

Authors' addresses: Paolo Fiallo and Andrea Clapasson, Centro Nazionale di Riferimento per la Malattia di Hansen e la Dermatologia Tropicale (CIRLEP), Dipartimento di Scienze della Salute, Università degli Studi di Genova, Viale Benedetto XV 7, 16132 Genova, Italy. Anna Favre and Carlo Pesce, Centro di Anatomia Patologica, DISTBIMO, Corso Europa 30, 16132 Genova, Italy.

Reprint requests: Carlo Pesce, Centro di Anatomia Patologica, DISTBIMO, Università di Genova, Corso Europa 30, 16132 Genova, Italy, Telephone: +39-010-3537400, Fax: +39-010-3537424, E-mail: pesce@unige.it.

REFERENCES

10. Brown LF, Olbricht SM, Berse B, Jackman RW, Matsueda G,


