Infectious Angiogenesis: *Bartonella bacilliformis* Infection Results in Endothelial Production of Angiopoetin-2 and Epidermal Production of Vascular Endothelial Growth Factor

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Pathological angiogenesis, the development of a microvasculature by neoplastic processes, is a critical component of the development of tumors. The role of oncogenes in the induction of angiogenesis has been extensively studied in benign and malignant tumors. However, the role of infection in inducing angiogenesis is not well understood. Verruga peruana is a clinical syndrome caused by the bacterium *Bartonella bacilliformis*, and is characterized by the development of hemangioma-like lesions, in which bacteria colonize endothelial cells. To gain insight into how this bacteria induces angiogenesis in *vivo*, we performed *in situ* hybridization of clinical specimens of verruga peruana for the angiogenesis factors vascular endothelial growth factor (VEGF), its receptors VEGFR1 and VEGFR2, and angiopoietin-2. High-level expression of angiopoietin-2 and VEGF receptors was observed in the endothelium of verruga peruana. Surprisingly, the major source of VEGF production in verruga peruana is the overlying epidermis. Infection of cultured endothelium with *B. bacilliformis* also resulted in induction of angiopoietin-2 in *vivo*. These findings imply a collaboration between infected endothelium and overlying epidermis to induce angiogenesis. (Am J Pathol 2003, 163:1321–1327)

Angiogenesis, the development of microvessels, is an indispensable component of normal physiological processes, including pregnancy, menstruation, organ formation, and wound repair.¹ ² These mechanisms have been co-opted by benign and malignant processes, as well as inflammatory processes. Oncogenes have been shown to induce the expression of angiogenic factors including vascular endothelial growth factor (VEGF).³ ⁴ Infections can also induce angiogenesis, and both viral and bacterial infections have been known to result in increased angiogenesis in *vivo*. The viral infections most commonly associated with increased angiogenesis *in vivo* include papovaviruses (SV40, human papillomavirus, polyoma), and herpesvirus (Epstein-Barr virus, human herpesvirus 8).⁵ ⁶ ⁷ These viral infections result in neoplasia and increased angiogenesis in part through viral-specific oncogenes, including SV40 large T antigen, polyoma middle T antigen, papillomavirus E6 and E7 genes, latent membrane protein 1 (LMP-1), and HHV8/KSHV-specific G proteins. The mechanisms through which bacterial infections cause angiogenesis is not well understood.⁸

Verruga peruana are lesions associated with human infection with *B. bacilliformis*, an arthropod transmitted disease endemic to the highlands of Peru.¹⁹ Infection with *B. bacilliformis* results in Oroya fever, a severe immunosuppressive infection characterized by high fever and anemia, which has a high mortality rate if untreated.¹² This disorder is also known as Carrion’s disease, after a Peruvian medical student, Daniel Carrion, who infected himself with verruga peruana tissue to prove the etiology of the disease.¹³ The convalescent state is associated with the development of hemangioma-like lesions which have been termed verruga peruana. These lesions ultrastructurally demonstrate endothelial colonization with *Bartonella* organisms.¹⁴ The mechanisms of induction of hemangioma-like lesions by *Bartonella* infection are not well understood, but has been postulated to be due to elaboration of a proangiogenic factor from infected endothelium. We demonstrate that infection of endothelium by *Bartonella* results in induction of angiopoietin-2 *in vitro* and *in vivo*. We found evidence of active angiogenesis in these lesions with high level expression of VEGFR1 and...
VEGFR2, which is observed in proliferative but not quiescent endothelium. However, the major source of the angiogenic factor VEGF is the suprabasal epidermis. These findings imply a collaboration between infected endothelial cells and overlying epidermis.

Materials and Methods

Infection of Human Umbilical Vein Endothelium Cell (HUVEC) with B. bacilliformis

HUVECs were obtained from Clonetics (San Diego, CA) and were routinely cultured in the manufacturer’s own medium (EGM2) supplemented with 2% fetal bovine serum. For experimental purposes, HUVECs (passage 2 to 4) were trypsinized and seeded on 1 mg/ml gelatin, pre-coated with tissue culture flask in M199 medium (Gibco) containing 20% fetal calf serum (FCS), heparin and bovine hypothalamus extract the day before infection. Cultures were maintained in a humidified atmosphere 5% CO2 and at 37°C. For infection purposes, HUVECs were incubated with approximately 100 bacteria per cell in M199 supplemented with 10% FCS for 26 hours. Negative controls had primary antibody substituted with buffer.

RNA Isolation and RT-PCR

Total RNA from Bartonella-infected and non-infected cell were isolated using the TRIzol Reagent Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Total RNA were dissolved in RNase-free water. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR System (Promega). For angiopoietin-1 the following primers were used: hAng1F, 5’-AGTCCAGAATCAGTGGAG-3’ and hAng1R, 5’-AGCAGCTGTATCTCAAGTCG-3’ (1 minute at 95°C, 1 minute at 59°C, 45 seconds at 72°C for 35 cycles). For VEGF receptor 1 (VEGFR1) the following primers were used: VEGFR1F, 5’-GATGTGAGAACAGAGGAATT-3’ and VEGFR1R, 5’-AAGCTAGTTTCTGAGGTG-3’ (1 minute at 95°C, 1 minute at 63°C, 45 seconds at 72°C for 35 cycles). For VEGF receptor 2 (VEGFR2) the following primers were used VEGFR2F, 5’-GATGTGAGAACAGAGGAATT-3’ and VEGFR2R, 5’-CATGGCTCTGCTTCTCCTTG-3’ (1 minute at 95°C, 1 minute at 62°C, 45 seconds at 72°C for 35 cycles).

In Situ Hybridization (ISH)

ISH was performed on 4-mm-thick sections of formalin-fixed, paraffin-embedded tissue, from four representative specimens of verruga peruana. Details of ISH have been reported previously. Briefly, slides were passaged through xylene and graded alcohols; 0.2 mol/L Tris/EDTA with 3 μg/ml proteinase K/0.2% glycine/4% paraformaldehyde in phosphate-buffered saline (pH 7.4), 0.1 mol/L triethanolamine containing 1/200 (v/v) acetic anhydride, and 2X SSC. Slides were hybridized overnight at 50°C with 35S-labeled riboprobes in the following mixture: 0.3 mol/L NaCl, 0.01 mol/L Tris (pH 7.6), 5 mmol/L EDTA 0.02% w/v Ficoll, 0.02% w/v polyvinylpyrrolidone, 0.02% w/v bovine serum albumin fraction V, 0.5% formamide, 10% dextran sulfate, 0.1 mg/ml yeast tRNA, 0.01 mol/L dithiothreitol (DTT). Post-hybridization washes included 2X SSC, 50% formamide, 10 mmol/L EDTA at 65°C; and 2X SSC. Slides were dehydrated through graded alcohols containing 0.3 mol/L ammonium acetate, dried, coated with Kodak NTB 2 emulsion and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 developer and the slides were counterstained with hematoxylin.

Immunohistochemistry

Unstained sections of formalin-fixed, paraffin-embedded tissue were immunostained with polyclonal antibodies against tie-2 (clone sc-324, 1/40; Santa Cruz Biotechnologies, Santa Cruz, CA) using an avidin-biotin-complex technique with the pressure-cooker heat-induced antigen retrieval and a DAKO Autostainer (DAKO, Carpinteria, CA). Negative controls had primary antibody substituted with buffer.
Results

*Bartonella* infection of endothelium has been shown to result in induction of endothelial proliferation through stimulation of mediators which have not been fully characterized. Given that VEGF production by endothelium appears to be produced primarily in malignant endothelial tumors (angiosarcomas) rather than benign tumors (hemangiomas), and that verruga peruana lesions are biologically benign, we felt it was unlikely that VEGF was the sole mediator of *Bartonella*-induced angiogenesis. Infection of endothelial cells with *B. bacilliformis* resulted in strong induction of angiopoietin-2 mRNA (Figure 1). Induction of other angiogenic molecules, such as VEGFR1, VEGFR2, and angiopoietin-1 were not observed (data not shown). To determine whether angiopoietin-2 is...
expressed in authentic human verruga peruana lesions, these lesions were subjected to in situ hybridization for angiopoietin-2, VEGF, VEGFR1, and VEGFR2.

Prominent expression of angiopoietin-2, tie-1, and tie-2 was observed in endothelial cells of verruga peruana by in situ hybridization (Figure 2). In addition, prominent endothelial expression of tie-2, the receptor for angiopoietin-2, was noted by immunohistochemistry on representative sections (Figure 3). Histological analysis of four representative verruga peruana lesions revealed epidermal hyperplasia and inflammation (Figure 4, A and B). In situ hybridization revealed prominent expression of VEGF in the suprabasal keratinocyte layer of verruga lesions but not normal skin (Figure 4, A and B). In situ hybridization revealed prominent expression of VEGF in the suprabasal keratinocyte layer of verruga lesions but not normal skin (Figure 4, A and B). Lesions were then examined for expression of VEGFR1 and VEGFR2, markers of angiogenic endothelium. High level expression of both VEGFR1 (Figure 4C) and VEGFR2 (Figure 4D) were observed in endothelial cells, confirming the presence of angiogenically active endothelium.

Discussion

Angiogenesis is a requirement for the maintenance of benign and malignant neoplasms, but its role in infectious disease is less well studied. While acute infection is often associated with tissue death, chronic infection is often associated with pathological tissue overgrowth and/or infiltration of host cells. Examples of this include endothelial proliferation in verruga peruana, bacillary angiomatosis, and Kaposi's sarcoma, granuloma formation in leishmaniasis and mycobacterial disease, lymphoid proliferation in Epstein-Barr virus infection, and keratinocyte hyperplasia in human papillomavirus infection.22

Oncogenic viruses most often encode viral-specific oncogenes which stimulate tumorigenesis and angiogenesis. In neoplasms induced by these agents, the primary source of angiogenic factors are the tumor cells themselves.9,23 However, in bacterial-induced angiogenesis, the source and identity of angiogenic factors are unknown. In the case of bacterial infections of endothelium, the causative organisms are often difficult to culture,24 and animal models do not exist that recapitulate the pathology seen in human disease. Because of this, it is imperative to study human tissue obtained from authentic human lesions.

Prior studies of human vascular lesions have demonstrated signal transduction aberrations in benign and malignant endothelium.3,25,26 Increased endothelial expression of angiopoietin-2 has been observed in hemangiomas of childhood, but the role of VEGF in hemangio-
mas is controversial, with some earlier studies detecting VEGF protein expression in hemangiomas, but later studies have failed to demonstrate endothelial expression of VEGF.\textsuperscript{27,28} Overexpression of VEGF in endothelial cells results in malignant transformation into angiosarcoma.\textsuperscript{21} The prior studies suggest that endothelial expression of VEGF is a sign of malignancy.\textsuperscript{29}

Prior studies have implicated that Bartonella-infected endothelium elaborate factors which stimulate angiogenesis.\textsuperscript{30} The identity of this factor(s) have not been previously elucidated. The finding of angiopoietin-2 in hemangiomas of childhood,\textsuperscript{28} along with the data showing that VEGF expression in endothelium is associated with malignancy prompted us to study whether Bartonella in-

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**Figure 4.** Expression of VEGF and VEGF receptors in verruga peruana lesions. A and B: Photomicrographs of hyperplastic epidermis overlying verruga peruana (A) and normal skin (B), showing strong expression of VEGF by suprabasal epidermal keratinocytes in verruga peruana, but low expression in normal skin (magnification, ×200). C and D: Expression of VEGFR1 and VEGFR2 in verruga peruana. Photomicrographs of a nodular focus of proliferating endothelial cells and surrounding inflammatory cells showing strong expression of VEGFR1 (C) and VEGFR2 (D) by endothelial cells.
duces angiopoietin-2. We found that *Bartonella* infection results in induction of angiopoietin-2 *in vitro* and *in vivo*, and human verruga peruana lesions express angiopoietin-2.

The role of angiopoietin-2 in angiogenesis has not been totally elucidated. Initially angiopoietin-2 was found to have antagonistic behavior to angiopoietin-1 and bind the same receptor, tie-2.31 These findings led investigators to believe that angiopoietin-2 may be an inhibitor of angiogenesis. However, later findings have demonstrated that angiopoietin-2 may stimulate tumor growth, and tumor cells overexpressing angiopoietin-2 have demonstrated highly malignant behavior and leaky vessels.32,33 Furthermore, hemangiomas of childhood have been demonstrated to express high levels of angiopoietin-2.28

Recently, *Bartonella* infection of endothelial cells *in vitro* has shown to lead to decreased apoptosis, which may account for the angiogenic phenotype.34 In addition, *Bartonella* infection has been shown to cause activation of the rac GTPase, and rac GTPase activation has been shown to stimulate angiogenesis and prevent apoptosis.16,35,36 Consistent with these findings, angiopoietin-2 activation of tie receptor signaling has been shown to result in activation of phosphoinositol-3 kinase/akt signaling, which are associated with both protection against apoptosis and stimulation of endothelial proliferation.37,38

Interestingly, we found that overlying epidermis is the primary source of VEGF in our lesions, and may serve as a trophic factor to maintain hemangioma-like lesions induced by *Bartonella* infection. Epidermal production of VEGF appears to be a common response to inflammatory stimuli in the skin, and may be due to elaboration of cytokines by the inflammatory process39 or through disruption of the epidermis by the verruga peruana lesions. Barrier disruption of the epidermis leads to induction of VEGF (Cerimele and Arbiser, unpublished data). We have observed that verruga peruana specimens express high levels of active mitogen-activated protein kinase (P-MAPK), as do hemangiomas of childhood.40 In contrast, malignant endothelial lesions (angiosarcoma), express low levels of activated MAP kinase, reflecting a decreased requirement for MAP kinase in tumorigenesis.40,41 Verruga peruana are highly angiogenic lesions, but are histologically benign. This is a fundamentally different pattern of angiogenesis compared with either virally-induced tumors or angiosarcomas (malignant endothelial tumors) in which the tumor cells themselves are the source of angiogenic factors.19,21,42 Our findings point to a novel form of angiogenesis in benign lesions, in which proliferation of endothelium and/or decreased apoptosis may be due to autocrine loops of angiopoietin-2 and tie-2, with paracrine contributions of VEGF produced outside the infected endothelium. The paracrine expression of VEGF may help maintain the lesions.

*Bartonella* infections are unique among bacteria in induction of benign hemangioma-like lesions. Currently, verruga peruana is an uncommon disease, but it histologically resembles the benign hemangioma of infancy, the most common neoplasm of children. This report is the first demonstration of a potential role of angiopoietin-2 in the pathogenesis of *Bartonella* infection, and demonstrates a novel mechanism of bacterial induction of angiogenesis.

References


