Activation of Hypoxia-Inducible Factor-1 in Bacillary Angiomatosis

Evidence for a Role of Hypoxia-Inducible Factor-1 in Bacterial Infections

Volkhard A.J. Kempf, MD*; Maria Lebiedziejewski*; Kari Alitalo, MD, PhD*; Joo-Hee Wälzlein; Urs Ehenthal; Jeannette Fiebig; Stephan Huber, PhD; Burkhardt Schütt, PhD; Christian A. Sander, MD; Steffen Müller, PhD; Guntram Grassl, PhD; Amir S. Yazdi, MD; Bernhard Brehm, MD; Ingo B. Autenrieth, MD

Background—Bartonella species are the only known bacterial pathogens causing vasculoproliferative disorders in humans (bacillary angiomatosis [BA]). Cellular and bacterial pathogenetic mechanisms underlying the induction of BA are largely unknown.

Methods and Results—Activation of hypoxia-inducible factor-1 (HIF-1), the key transcription factor involved in angiogenesis, was detected in Bartonella henselae–infected host cells in vitro by immunofluorescence, Western blotting, electrophoretic mobility shift, and reporter gene assays and by immunohistochemistry in BA tissue lesions in vivo. Gene microarray analysis revealed that a B henselae infection resulted in the activation of genes typical for the cellular response to hypoxia. HIF-1 was essential for B henselae–induced expression of vascular endothelial growth factor as shown by inhibition with the use of HIF-1–specific short-interfering RNA. Moreover, infection with B henselae resulted in increased oxygen consumption, cellular hypoxia, and decreased ATP levels in host cells. Infection with a pilus-negative variant of B henselae did not lead to cellular hypoxia or activation of HIF-1 or vascular endothelial growth factor secretion, suggesting a crucial role of this bacterial surface protein in the angiogenic reprogramming of the host cells.

Conclusions—B henselae induces a proangiogenic host cell response via HIF-1. Our data provide for the first time evidence that HIF-1 may play a role in bacterial infections. (Circulation. 2005;111:1054-1062.)

Key Words: angiomatosis, bacillary | Bartonella henselae | angiogenesis | HIF-1 protein | hypoxia

Angiogenesis is a multistep process resulting in the formation of new blood vessels from preexisting vasculature. Newly formed vessels supply oxygen and nutrients to growing tumors and are necessary for tumor progression and metastasis.1 Angiogenesis is also a component of various cardiovascular and inflammatory diseases.2 Hypoxia-inducible factor-1 (HIF-1) is a key transcription factor for the induction of angiogenic growth factors that adjust the vascular oxygen supply to tissue metabolic demands.3,4 Of the many genes induced by HIF-1, vascular endothelial growth factor (VEGF) plays a critical role in triggering angiogenesis as the major hypoxia-inducible mitogen for endothelial cells.5 Interestingly, human herpesvirus-8 (HHV-8) and several Bartonella species induce angiogenesis in humans. HHV-8 causes the vasculoproliferative disorder Kaposi’s sarcoma,6 which has a high frequency among immunocompromised patients, such as those infected with HIV. HHV-8–infected cells express VEGF on HIF-1 activation,7 and this mechanism was implicated in endothelial cell proliferation in the lesions.8 Bartonella henselae and B quintana are the etiologic agents of bacillary angiomatosis (BA) and bacillary peliosis (BP), which are histologically characterized as lobulated proliferations of mainly capillary-sized vessels and predominantly affect HIV patients.9 These slow-growing bacteria are facultative intracellular pathogens that, like HHV-8, also induce VEGF in host cells in vitro and in BA or BP lesions of patients.10 Endothelial cells are one presumed habitat of Bartonella.11 Dissecting the angioproliferative strategies used

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From the Institut für Medizinische Mikrobiologie und Hygiene, Eberhard-Karls Universität, Tübingen, Germany (V.A.J.K., M.L., J.W., U.E., J.F., S.M., G.G., I.B.A.); Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland (K.A.); Institute of Physiology, Tübingen, Germany (S.H.); Universitätsklinik für Kinderheilkunde und Jugendmedizin, Tübingen, Germany (B.S.); Klinik für Dermatologie und Allergologie, Ludwig Maximilians Universität, Munich, Germany (C.A.S., A.S.Y.); and Medizinische Universitätsklinik III, Tübingen, Germany (B.B.).

*The first 3 authors contributed equally to this work.

The online-only Data Supplement, which contains Figures 1 through V and a table, can be found with this article at http://www.circulationaha.org.

Correspondence to Dr Volkhard A.J. Kempf, Institut für Medizinische Mikrobiologie und Hygiene, Elfriede-Aulhorn-Strasse 6, D-72076 Tübingen, Germany. E-mail volkhard.kempf@med.uni-tuebingen.de

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by *B. henselae* is highly important in terms of understanding bacterial pathogenicity, angiogenesis, and pathogen-triggered tumor formation in humans.

### Methods

**Bacteria and Cell Culture**

*B. henselae* Marseille, *Legionella pneumophila* (ATCC 33216), HeLa229, and human umbilical vein endothelial cells (HUVECs) were infected as described.10 SB202190 (p38 inhibitor), PD98059 (MEK1 inhibitor), and parthenolide (nuclear factor-κB [NF-κB] inhibitor12) were purchased from Calbiochem; tumor necrosis factor-α (TNF-α) was purchased from R&D Systems.

**Immunostaining and Confocal Laser Scanning Microscopy**

Differential staining of extracellular and intracellular *B. henselae* and confocal laser scanning microscopy were performed as described.11

**Transmission Electron Microscopy**

Transmission electron microscopy was performed as described.10

**Lipopolysaccharide Preparation**

Lipopolysaccharide (LPS) was prepared as described.13

**RNA Isolation and Microarray Analysis**

Cells (2×10^6) were grown for 24 hours and infected with *B. henselae*. Total RNA was extracted with the use of the RNeasy mini kit.

### Upregulated Genes in HeLa Cells 6 Hours on *B. henselae* Infection

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene Abbreviation</th>
<th>Induction Light Cycler†</th>
<th>Transcription Factor</th>
<th>Biological Function</th>
<th>U95 Array Designations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Interleukin-8 IL-8</td>
<td>6.41</td>
<td>5.80†</td>
<td>NF-κB</td>
<td>35372_r_at</td>
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<tr>
<td>2.</td>
<td>Stanniocalcin-2 STC2</td>
<td>5.18</td>
<td>Yes‡</td>
<td>HIF-1</td>
<td>32043_at</td>
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<tr>
<td>3.</td>
<td>Adrenomedullin ADM</td>
<td>3.88</td>
<td>Yes‡</td>
<td>HIF-1</td>
<td>34777_at</td>
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<tr>
<td>4.</td>
<td>Ephrin A1 EFNA1</td>
<td>3.74</td>
<td>ND</td>
<td>HIF-1</td>
<td>40425_at</td>
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<tr>
<td>5.</td>
<td>Vascular endothelial growth factor VEGF</td>
<td>3.54</td>
<td>5.10†</td>
<td>HIF-1</td>
<td>1953_at, 36100_at, 36101_at</td>
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<td>6.</td>
<td>Insulinlike growth factor binding protein-3 IGFBP-3</td>
<td>2.67</td>
<td>Yes‡</td>
<td>HIF-1</td>
<td>37319_at, 1586_at</td>
</tr>
<tr>
<td>7.</td>
<td>Endothelin-2 ET-2</td>
<td>2.13</td>
<td>ND</td>
<td>HIF-1</td>
<td>1092_at</td>
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**Glycolysis**

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<th>Transcription Factor</th>
<th>Biological Function</th>
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<tr>
<td>8.</td>
<td>Hexokinase-2 HK2</td>
<td>4.37</td>
<td>Yes‡</td>
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<td>40964_at</td>
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<td>9.</td>
<td>Glucose transport protein-3 SLC2A3</td>
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<td>ND</td>
<td>HIF-1</td>
<td>Glucose transport into cells, 36979_at</td>
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<td>10.</td>
<td>Enolase-2 ENO2</td>
<td>2.82</td>
<td>ND</td>
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<td>40193_at</td>
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**Signal transduction**

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<th>Biological Function</th>
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<tr>
<td>11.</td>
<td>N-myc downstream regulated gene-1 NDRG1</td>
<td>4.85</td>
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<td>12.</td>
<td>Musculoaponeurotic fibrosarcoma oncogene homolog f MAFF</td>
<td>4.45</td>
<td>ND</td>
<td>?</td>
<td>Transcription from Pol2 promoter, 36711_at</td>
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<td>13.</td>
<td>Nuclear factor IL-3 regulated NFIIL3</td>
<td>2.90</td>
<td>ND</td>
<td>HIF-1§</td>
<td>Transcription from Pol2 promoter, 37544_at</td>
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<td>14.</td>
<td>Cyclin G2 CCNG2</td>
<td>2.77</td>
<td>Yes‡</td>
<td>HIF-1</td>
<td>37544_at, 1913_at</td>
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<td>15.</td>
<td>Hyperpolarization activated cyclic nucleotide gated potassium channel-2 HCN2</td>
<td>2.68</td>
<td>ND</td>
<td>?</td>
<td>Signaling, 34520_at</td>
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<td>16.</td>
<td>Max-interacting protein-1 MXI1</td>
<td>2.51</td>
<td>ND</td>
<td>?</td>
<td>Control of cell cycle, 654_at</td>
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<td>17.</td>
<td>p21-activated kinase-2 PAK2</td>
<td>2.26</td>
<td>ND</td>
<td>HIF-1§</td>
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**Unknown**

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<th>Biological Function</th>
<th>U95 Array Designations</th>
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<td>18.</td>
<td>KIAA0742 protein KIAA0742</td>
<td>3.70</td>
<td>Yes‡</td>
<td>?</td>
<td>Unknown, 34786_at</td>
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<td>19.</td>
<td>Homo sapiens cDNA FLJ22182c</td>
<td>2.85</td>
<td>Yes‡</td>
<td>?</td>
<td>Unknown, 40079_at</td>
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<tr>
<td>20.</td>
<td>Hypothetical protein FLJ20500</td>
<td>2.59</td>
<td>ND</td>
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<td></td>
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*ND indicates not determined.*

*Analysis of 2 independent data sets was performed on Affymetrix Microarray suite 5.0 and Affymetrix data mining tool 3.0 as described in Methods.

†Fold induction (Light Cycler) compared with negative control (VEGF: β1-microglobulin normalized; IL-8: GAPDH normalized).

‡Detected with conventional RT-PCRs.

§Indirect evidence (upregulation on hypoxia).

||Contains HIF-1–responsive element (NM_9506686).
Reverse Transcription of mRNA and Polymerase Chain Reactions

The reverse transcription (RT) reactions (HeLa cells, HUVECs) were performed with the use of the SuperScript ds-cDNA synthesis kit (Invitrogen) and oligo-dT₃₀ primers. The adenomucedulin (ADM), insulinlike growth factor binding protein-3 (IGFBP-3), stanniocalcin-2 (STC2), hexokinase-2 (HK2), cyclin G2 (CCNG2), N-myc downstream regulated gene-1 (NDRG1), KIAA0742 protein (KIAA0742), Homo sapiens cDNA (FLJ21282h1s), interleukin-1β (IL-1β), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF receptor-2 (VEGFR-2), and β-actin cDNAs were amplified by conventional RT-PCR (see the Data Supplement Table). Quantification of VEGF and interleukin-8 (IL-8) mRNA (LightCycler Primer Sets, Search-LC) was performed by real-time PCR with a LightCycler System (Roche).

Immunooassays and Western Blotting

VEGF, IL-8, IGFBP-3, and ADM concentrations were measured from cell culture supernatants as described. For Western blot analysis of HK2, polyclonal antibodies (SantaCruz) were used. Phosphorylation of p38 and p42/44 mitogen-activated protein (MAP) kinases was evaluated with the use of a phospho-MAP kinase p38 and p42/44 kit (Cell Signaling).

Detection of HIF-1α by Immunofluorescence and Western Blotting

For HIF-1α immunostaining, HeLa cells were seeded on coverslips and infected with B. henselae or exposed to hypoxia. Immuno- staining of HIF-1α was performed with the use of anti-HIF-1α (Novus) and TRITC-conjugated anti-IgG antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

For HIF Western blotting, cellular proteins were extracted, subject to the Bradford assay (BioRad), blotted onto polyvinylidene difluoride membranes, and incubated with HIF-1α-specific (Becton Dickinson) or HIF-2α-specific (Novus) antibodies.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from HeLa cells as described, and protein concentrations were assessed via the Bradford assay (Bio- rad). Oligonucleotide probes (NF-κB consensus [NF-κB 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Santa Cruz], HIF-1α consensus [HIF-1α 5'-GCC GCA CTT GCT GTC TCA-3', Metabion] were labeled with [γ³²P]ATP (Amersham). In competition experiments, extracts were incubated with the labeled probes in the presence of a 100-fold excess of unlabeled oligonucleotides. Antibodies against p50 (Santa Cruz) were included in the binding reaction for supershift analysis.

DNA Constructs, Transient Transfection, and Determination of Reporter Gene Activity

For reporter gene assays, a VEGF promoter/luciferase reporter construct (pVEGF.4) was used. For normalization, cotransfections were performed with the use of pCMV β-galactosidase (pCMV β-gal; Clontech). HeLa cells were transiently transfected with the use of ExGen500 (Fermentas) and incubated for 24 hours at 37°C. Transfected cells were infected with B. henselae or exposed to hypoxia. After 36 hours, cells were lysed for determination of Luc activity, protein determination, and measurement of β-galactosidase activity and total protein concentration. Every experiment was done in quadruplicate.

The degree of induction was determined as the ratio of Luc activity of B. henselae–infected or hypoxia-exposed cells to that of uninfected control cells.

Gene Silencing Using HIF-1α Short-Interfering RNA

Cells were seeded in 6-well plates, and the short-interfering RNA (siRNA) duplexes [HIF-1α sense r(CUGAUGACCCAGCAAUCUGA)d(TT), antisense r(UCAAGUUAGCUUCAUCAG)d(TT)]\(^\text{15}\); control sense r(UUCUCGGAACGUUCAGCU)d(TT), antisense r(CGUGACACGUUCGGAA)d(TT)]\(^\text{15}\) were diluted at a final concentration of 200 nmoL/L. RNAiFect (Qiagen) was used for transfection. Cell culture medium was changed, and the cells were incubated overnight before infection.

Figure 1. Induction of host cell transcripts and proteins on B. henselae infection in HeLa cells. HeLa cells were infected with B. henselae (B. h.), and total RNA was extracted 6 hours after infection and transcribed into cDNA. Gene induction was assessed by real-time PCR (VEGF: 5.2-fold induction; IL-8: 5.8-fold induction) or RT-PCR (ADM, IGFBP-3, HK2, β-actin, duplicates). For determination of secreted protein levels, cells were infected, and supernatants (triplicates) were analyzed 48 hours after infection via ELISA (VEGF, IL-8: pg/mL) or radioimmunoassay (ADM: pg/mL; IGFBP-3: ng/mL). For Western blotting (HK2, duplicates), cellular extracts were prepared 8 hours after infection (loading control: actin). C indicates control uninfected cells.
Immunohistochemistry

Sections of histologically confirmed paraffin-embedded specimens of BA were stained with the use of specific anti-HIF-1α antibodies (Novus) and the alkaline phosphatase/anti–alkaline phosphatase technique with hematoxylin counterstaining. Unaffected skin was used as control.

Detection of Cellular Hypoxia and Oxygen Consumption

Cellular hypoxia was detected by adding pimonidazole hydrochloride (200 mmol/L; Hypoxyprobe-1, Chemicon) to HeLa cells that were subsequently infected or exposed to hypoxia for 4 hours. Staining was performed according to the manufacturer’s instructions.

The dissolved O2 concentration was directly measured in the medium of B henselae–infected and control cell cultures (for details, see Data Supplement Figure I). An O2-tight closed chamber was built around the head of a Clark electrode (Conrad Electronics). HeLa cells grown on coverslips were infected for 4 hours with B henselae and placed in the chamber filled with RPMI 1640 medium saturated with CO2 (5%) and O2 (20.8%). The oxygen electrode was allowed to equilibrate for 1 minute at 37°C followed by a 10-minute period of oxygen measurement. O2 concentration (mg/L) was determined at 60-second intervals.

Measurement of Intracellular ATP

For quantification of cellular ATP, HeLa cells were infected with B henselae or exposed to hypoxia for 4 hours, and the intracellular ATP was determined with the use of the CLSII Assay Kit (Roche). ATP levels were normalized to the total protein concentration of the cellular extracts.

Statistical Analysis

All data shown are from representative experiments. Differences between mean values were analyzed with the Student t test. P<0.05 was considered statistically significant.

Figure 2. Activation of HIF-1 on B henselae (B. h.) infection in HeLa cells. a, HIF-1 activation was detected 6 hours after infection by immunofluorescent staining with the use of HIF-1α–specific and TRITC-labeled secondary antibodies (top row). Nuclei and bacteria were stained with DAPI (bottom row). Bar=20 μm. b, HIF-1α protein (120 kDa) was analyzed in Western blots 6 hours after infection in duplicates (loading control: actin). c, Induction of the VEGF promoter. HeLa cells were transfected with a VEGF promoter/luciferase reporter construct (pVEGF.4) 24 hours before infection, and induction was determined by chemiluminescence 36 hours after infection. C indicates control uninfected cells; H, hypoxia. d, Nuclear localization of transcription factor HIF-1α probe in the presence of a 100-fold excess of an unlabeled competitor probe (comp.). e, Effect of HIF-1α siRNA on HIF-1 activation on infection. HIF-1α was analyzed in Western blots 6 hours after infection (loading control: actin). f, Effect of HIF-1α siRNA on VEGF expression on infection. VEGF (pg/mL) was quantified in cell culture supernatants 48 hours after infection. C indicates control uninfected cells; NS, non-sense control siRNA; HIF-1α, HIF-1α siRNA. *Significant difference compared with B henselae–infected cells and with B henselae–infected cells treated with control siRNA (P<0.05).

Results

B henselae Induces a Proangiogenic Gene Program

For analysis of the genomic response to B henselae infection, infected and uninfected HeLa cells were compared in RNA microarray analysis 6 hours after infection. Confocal laser scanning microscopy showed that at this time >99% of the cells contained bacteria (Data Supplement Figure II). Among ~12 000 genes analyzed, 20 genes were found to be upregulated >2-fold on infection. As indicated in the Table, at least 7 of these genes (eg, IL-8, ADM, ephrin A1 [EFNA1], VEGF) are known to play a role in angiogenesis and vessel maturation, 3 are involved in glycolysis, and 7 are involved in signal transduction and cell growth. The function of 3 of the induced genes is currently unknown. Gene downregulation was not detected. Of the 20 genes, 14 of are known to be directly or indirectly regulated via HIF-1, suggesting that the major trigger of angiogenic gene induction on B henselae infection was HIF-1. IL-8 was an exception; this highly induced gene is strongly induced by NF-κB but not by HIF-1. Quantitative real-time PCR or semiquantitative RT-PCR was used to confirm results of the gene array experiments (Figure 1, Data Supplement Figure III). As a test for possible false-negative results, selected NF-κB–regulated genes (ICAM-1, MCP-1, IL-1β, GM-CSF) or the HIF-2–regulated VEGFR-2 gene were not affected on B henselae infection. Moreover, increased protein expression was shown for 5 of the induced genes (VEGF, IL-8, IGFBP-3, ADM, and HK2; Figure 1). Results were consistent with the microarray data indicating a significant change toward a proangiogenic gene program in host cells.
**B henselae** Induces HIF-1, Cellular Hypoxia, and Decrease of ATP

The induced gene pattern suggested that HIF-1 activation has a crucial role in the host cell response to *B henselae* infection. We therefore investigated the activation of HIF-1 by *B henselae* in HeLa cells (Figure 2). Immunofluorescence analysis revealed the appearance of nuclear HIF-1α 6 hours after *B henselae* infection, similar to hypoxia-treated cells (Figure 2a), and consistent results were obtained by Western blotting (Figure 2b). Transfection experiments with the use of a VEGF promoter/luciferase reporter that is specifically regulated by HIF-1 revealed that *B henselae* increases VEGF gene transcription ∼4-fold compared with ∼8-fold stimulation by hypoxia (Figure 2c). Nuclear HIF-1α activation was also demonstrated by electrophoretic mobility shift assay (EMSA), which indicated the enhanced capacity of the induced HIF-1α complex to bind to its target consensus DNA sequences (Figure 2d). Finally, activation of HIF-1 and subsequent induction of VEGF secretion after *B henselae* infection was completely inhibited when the cells were treated with HIF-1α siRNA before infection (Figure 2e, 2f). These results clearly indicate that HIF-1 is activated on a *B henselae* infection and that the increased VEGF secretion on *B henselae* infection is dependent on HIF-1.

Endothelial cells that were heavily infected by *B henselae* (Data Supplement Figure II) also upregulated VEGF, IL-8, ADM, and HK2 after *B henselae* infection (Figure 3). Moreover, VEGFR-2, known to be regulated via HIF-1 and subsequent induction of VEGF secretion after *B henselae* infection was completely inhibited when the cells were treated with HIF-1α siRNA before infection (Figure 2e, 2f). These results clearly indicate that HIF-1 is activated on a *B henselae* infection and that the increased VEGF secretion on *B henselae* infection is dependent on HIF-1.

Because of the strong IL-8 induction, we also confirmed NF-κB activation by EMSA (Figure 4a). To dissect the NF-κB–regulated IL-8 from the HIF-1–regulated proangiogenic response, the infection process was modulated by the NF-κB inhibitor parthenolide12 (Figure 4b). This inhibited IL-8 secretion from *B henselae*–infected cells completely but had only little effect on VEGF secretion. This and the data obtained with HIF-1α siRNA strongly suggest that NF-κB is responsible for IL-8 induction, whereas HIF-1 triggers VEGF induction. The MAP kinases p38 and p42/44, which were rapidly and persistently phosphorylated in *B henselae*–infected cells (Figure 4c), contribute only little to the *B henselae*–induced VEGF secretion because preincubation with PD98059 (p42/44 inhibitor) or SB202190 (p38 inhibitor) caused only a slight reduction of the VEGF levels (Figure 4d). These results suggest that activation of HIF-1 on a Bartonella infection occurs via hypoxic activation, presumably involving inhibition of prolyl and asparaginyl hydroxylases.4

Because hypoxia is known to be a potent activator of HIF-1,6,7 we investigated whether infection with *B henselae* leads to an increased O2 consumption and cellular hypoxia. Experiments quantifying the dissolved O2 concentration in a closed cell culture chamber and with the use of a hypoxia-sensitive dye indicated that both processes indeed occurred (Figure 5a to 5c). O2 consumption was increased ∼9-fold in the *B henselae*–infected cells. Cellular hypoxia did not result from bacterial overgrowth because the pH of cell culture supernatants was unaffected in the same experiments (control 7.52, *B henselae* 7.51, hypoxia 7.28). In addition, infection resulted in decreased ATP levels in host cells (Figure 5d).

![Figure 3. Endothelial cell response to *B henselae* (B. h.) infection.](image)

- **a.** Induction of host cell transcripts and proteins on *B henselae* infection in HUVECs. RNA was extracted 6 hours after infection and transcribed into cDNA. Gene induction was assessed by real-time PCR (VEGF: 11.9-fold induction; IL-8: 4.2-fold induction) or RT-PCR (ADM, HK2, KDR, MCP-1, GM-CSF, β-actin, duplicates). For determination of secreted protein levels, cells were infected, and supernatants (triplicates) were analyzed 48 hours after infection via ELISA (VEGF, IL-8: pg/ml). b. Activation of HIF-1 and HIF-2 on *B henselae* infection in HUVECs. HIF-1α protein (120 kDa) and HIF-2α protein (118 kDa) were analyzed in Western blots 6 hours after infection (loading control: actin). Cont. indicates control; n.d., not determined.
Expression of *B. henselae* Pili Is Crucial for Activation of HIF-1

To analyze whether pilus expression determines the activation of HIF-1, HeLa cells were infected with wild-type (wt) or pilus-negative (Pil-/H11002) *B. henselae* (Figure 6a). Analysis of LPS composition disclosed no differences between both strains (Figure 6b). Pilus expression was crucial for activation of HIF-1 (Figure 6c), VEGF expression (Figure 6d), and cellular hypoxia (Figure 6e), suggesting that pili are necessary for inducing an angiogenic host cell response by *B. henselae*. Moreover, HIF-1 activation was not observed when cells were exposed to *B. henselae* LPS or when infected with *L. pneumophila* (Data Supplement Figures IV and V).

**Figure 4.** Activation of NF-κB and role of MAP kinases (p38, p42/44) on *B. henselae* (*B. h.*), infection in HeLa cells. NF-κB was detected 2 hours after infection by EMSA (a). In competition, experiments employed a 100-fold excess of the unlabeled competitor probe (comp.). p50 and p65/c-Rel are NF-κB subunits. Supershifts were performed with the use of p50 subunit specific antibodies. b, HeLa cells were treated with parthenolide (5 μmol/L, 20 μmol/L) 30 minutes before infection. VEGF and IL-8 production (pg/mL) were determined by VEGF ELISA in supernatants collected 48 hours after infection. Note that the NF-κB inhibitor parthenolide abolishes secretion of IL-8 on *B. henselae* infection completely, whereas secretion of VEGF is much less decreased. c, Phosphorylation of p38 and p42/44 was analyzed in duplicates 30 minutes after *B. henselae* (*B. hens.*), infection (positive control: TNF-α, 50 ng/mL) by Western blotting with anti-phospho-p38- or -p42/44-specific antibodies (pp38, pp42/44). Blots were stripped and reprobed with anti-p38 or -p42/44 antibodies (p38, p42/44). d, HeLa cells were treated with the p38 inhibitor SB202190 (5 μmol/L) or the p42/44 inhibitor PD98059 (25 μmol/L) 30 minutes before infection.

**Figure 5.** Consumption of O₂, cellular hypoxia, and ATP depletion occurs on *B. henselae* infection. a, O₂ concentration (conc.) time course in the medium of control (C) and infected HeLa cells (4 hours after infection) as measured in a closed medium-filled chamber by using a Clark O₂ electrode. Data are normalized to the 1-minute value on sealing of the chamber. b, O₂ consumption (consumpt) was calculated for 10⁵ cells by linear regression of the O₂ concentration decrease between 1 and 10 minutes of recording (data from a; mean±SE; n=8). c, Hypoxia (H) of HeLa cells was detected 4 hours after infection by the use of pimonidazole hydrochloride. Immunofluorescent staining was performed with the use of monoclonal pimonidazole-specific antibodies and TRITC-labeled secondary antibodies (top row). Nuclei and bacteria were stained with DAPI (bottom row). Bar=20 μm. d, Intracellular ATP contents 4 hours after infection or hypoxia. ATP levels were normalized to the total protein (prot) concentration of the cellular extracts.

**HIF-1 Is Activated in BA Lesions From Patients**

To investigate whether HIF-1 activation also occurs in vivo in *Bartonella*-infected tissues, sections from BA lesions were stained for HIF-1α (Figure 7). Nuclear HIF-1α was stained...
strongly in cells identified as histiocytes or macrophages infiltrating BA lesions. These observations are consistent with previously published results of HIF-1α staining in hypoxic tumor tissues\textsuperscript{17} and with recent data showing that HIF-1α is crucial in myeloid cell functions associated with inflammation and bacterial defense functions.\textsuperscript{24} From this data we conclude that HIF-1 induction occurs in *B. henselae* infections both in vitro and in vivo.

**Discussion**

Our present studies reveal a novel mechanism of angiogenesis associated with a bacterial infection. We show here that infection with *B. henselae*, the causative agent of angiogenic proliferation of capillary vessels in BA, results in HIF-1 activation and leads to a gene expression pattern typical for the cellular response to hypoxia. In accordance, nuclear HIF-1 was detected in *Bartonella*-infected cells in vitro and in vivo in BA patient samples. Our results suggest that activation of a HIF-1–dependent cell response on a *Bartonella* infection causes the vasculoproliferative disorder in BA and BP.

Angiogenesis research has been focused mainly on cancer and cardiovascular diseases.\textsuperscript{1} The HIF-1–induced angiogenic gene reprogramming would indeed be an excellent target for therapeutic intervention in solid tumors, including hemangioblastomas, and also in Kaposi’s sarcoma.\textsuperscript{4,7} On the other hand, activation of the HIF pathway could be of therapeutic importance in infections like BA, where the host response needs to be modulated to avoid excessive angiogenesis. The findings presented in this study could be relevant for the development of novel therapeutic strategies against BA and other infections associated with angiogenesis.

**Figure 6.** Influence of *B. henselae* pili on HIF-1 activation, VEGF secretion, and cellular hypoxia. a, Pili expression of Columbia agar–grown *B henselae* determined by microscopy. wt indicates wild-type; Pil−, pilus negative. b, LPS composition of *B henselae* wt and Pil− analyzed in silver-stained gels. c, Activation of HIF-1 analyzed in Western blots 6 hours after infection (duplicates; loading control: actin). *B. h.* indicates *B henselae*. d, Expression of VEGF quantified in cell culture supernatants 48 hours after infection. e, Hypoxia of HeLa cells was detected 4 hours after infection by the use of pimonidazole hydrochloride and TRITC-labeled secondary antibodies (top row). Nuclei and bacteria were stained with DAPI (bottom row). Bar=20 μm.

**Figure 7.** HIF-1α is activated in BA patient’s samples. Immunohistochemical detection of HIF-1α in unaffected skin (a) and in 2 histologically confirmed patient’s specimens of BA (b, c) with the use of an HIF-1α–specific antibody (red color). HIF-1α staining colocalizes with the nuclei in both BA samples (arrows).
value in tissue ischemia associated with cardiovascular diseases. However, HIF activation in bacterial infections has not been described previously, although genetic deletion of HIF-1α in myeloid cells compromises inflammatory responses. Bartonella species are the only known bacterial pathogens causing vasculoproliferative disorders in humans. VEGF induction was described in B henselae infections in vivo and in vitro, and a role has also been suggested for macrophage-secreted angiogenic agents.

Only 20 genes were found to be significantly upregulated on B henselae infection in our microarray analysis. Of these, 14 are known HIF targets. Several of these were confirmed by independent assays, including VEGF, which is known to be a key factor in the triggering of angiogenic processes, and STC2, ADM, IGFBP-3, and HK2, which have also been implicated in hypoxic cellular responses or angiogenic processes. IL-8, which is apparently the only NF-κB-regulated gene in our B henselae infection model of HeLa cells, is also known to be a strong vasculoproliferative agent. We hypothesize that the induced growth factors and cytokines secreted by the infected host cells contribute to an angiogenic cocktail responsible for endothelial proliferation in BA or BP, whereas the induction of HIF-regulated glycolytic genes may be linked to the metabolic demands of the B henselae–infected host cells (Figure 8).

Interestingly, some of the induced genes have been reported to be upregulated via HIF-1 or hypoxia in various types of human tumors (eg, enolase-2 [ENO2], NDRG-1, musculoaponeurotic fibrosarcoma oncogene homolog f [MAFF], nuclear factor IL-3 regulated [NFIL3], cyclin G2 [CCNG2], p21-activated kinase-2 [PAK2], and the hypothetical protein FLJ20500). The gene expression pattern in Bartonella-infected cells thus shows striking similarities with that in other angiogenesis models. Of interest is the fact that besides the angiogenic cytokine IL-8, the response in HeLa cells lacked the typical proinflammatory genes that are found to be induced via NF-κB in other bacterial infections. However, the induction of a broad spectrum of proinflammatory genes and cytokines could be deleterious for the facultative intracellular bacteria. In accordance, we did not find a broad activation of NF-κB–regulated proinflammatory cytokines. These cytokines are commonly induced on bacterial infection of endothelial cells, possibly because of LPS-mediated signals. However, HeLa cells, in contrast to endothelial cells, do not express toll-like receptor-4, which is required for these signals. Finally, the HIF-2 activation in HUVECs is a cell type–specific response that upregulates VEGFR-2 and may amplify the VEGF-driven angiogenic loop by making endothelial cells more sensitive to the secreted VEGF.

HIF-1 activation was observed in vitro and in patient samples from BA. This is consistent with our previous observations regarding increased VEGF expression in BA and BP lesions. Macrophages that infiltrate BA lesions were highly positive for HIF-1α and they are known to secrete VEGF on Bartonella infection in vitro. How HIF-1 is activated on a B henselae infection is not yet clear. The intracellular bacteria should consume a lot of oxygen, and the resulting hypoxia is known to inhibit the prolyl hydroxylases that target HIF-1α to ubiquitin-mediated degradation. In fact, infection with B henselae resulted in increased O2 consumption and hypoxia in the host cells and was accompanied by an increased consumption of cellular ATP. Furthermore, the MAP kinases p42/44 and p38 were phosphorylated on B henselae infection. However, although these kinases have been reported to enhance the transcriptional activity of HIF-1 by phosphorylation (p42/44) and increase VEGF mRNA stability (p38), these specific MAP kinase inhibitors induced only a small decrease of the B henselae–triggered VEGF secretion in our model. Stabilization of HIF-1α on a B henselae infection could thus occur by a mechanism similar to that in cells exposed to hypoxia.

Interestingly, the B henselae Pil’ variant did not induce cellular hypoxia, activation of HIF-1, or VEGF expression (Figure 6). Recently, we identified this “pilus” as the non-fimbrial adhesin BadA. When one considers this and the
observation that HIF-1 activation does not occur in LPS-exposed cells, it is highly unlikely that LPS is involved in the activation of HIF-1 by B henselae. Additionally, HIF-1 activation did not occur when cells were infected with L pneumophila. This is not surprising because L pneumophila infections do not result in vasculoproliferative disorders but instead in inflammatory disease. Our results suggest that Bartonella-specific bacterial mechanisms are necessary for the induction of the angiogenic response. The observation that Bartonella induces an HIF–1–driven cellular phenotype is exciting and provides a new aspect to angiogenesis research. Whether similar strategies are shared by other bacterial pathogens needs to be elucidated further.

Acknowledgments

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References

Activation of Hypoxia-Inducible Factor-1 in Bacillary Angiomatosis: Evidence for a Role of Hypoxia-Inducible Factor-1 in Bacterial Infections
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Supplementary information

Supplementary Table 1. Primers and PCR conditions.

Supplementary Fig. 1. Details on the oxygen-tight closed chamber. (a) An oxygen-tight closed chamber was built around the head of a Clark electrode and placed on a magnetic stirrer in a cell culture incubator (37° C). (b) Clark electrode and oxygen-tight closed chamber in more detail. (c) The oxygen tight cell culture chamber in detail. HeLa cells (grown overnight on coverslips) were placed in the bottom of this chamber filled with CO₂ (5%) and O₂ (20,8 %)-saturated RPMI 1640 medium. A nylon mesh was mounted on top of the cells to allow magnetic stirring of the medium without destruction of the cell layer. (d) Schematic draft of the chamber.

Supplementary Fig. 2. Course of B. henselae infection analysed by confocal laser scanning microscopy 1, 3 and 6 hours after infection of HeLa cells and HUVECs. Extracellular bacteria were labeled by fluorescein-isothiocyanate (FITC)-conjugated antibodies (green signal), intracellular bacteria were labeled by Cy-5-conjugated antibodies (blue signal). Filamentous actin was stained with tetramethylrhodamine-isothiocyanate (TRITC)-labeled phalloidin (red signal). Scale bar: 20 µm.

Supplementary Fig. 3. Induction of host cell transcripts upon B. henselae infection. HeLa cells were infected with B. henselae (B.h.), total RNA was extracted six hours after infection and transcribed into cDNA. (a) Gene induction was assessed in duplicate by RT-PCRs (STC2, CCNG2, NDRG1, FLJ22182fis, KIAA0742). (b) Genes not regulated in HeLa cells upon infection. Cells were infected with B. henselae (B.h.) or stimulated with PMA. Gene induction was assessed by RT-PCRs in duplicate (ICAM-1, MCP-1, IL-1β, GM-CSF, VEGFR-2, β-actin). C: control uninfected cells.

Supplementary Fig. 4. Failure of HIF-1 activation by LPS. LPS from B. henselae was added to HeLa cells (1000 ng/ml) and activation of HIF-1 was analysed in Western blots six hours after infection (duplicates are shown; loading control: actin).
Supplementary Fig. 5.: Failure of HIF-1 activation by *L. pneumophila* in HeLa cells. Activation of HIF-1 analysed in Western blots six hours after infection (duplicates are shown; loading control: actin). Note that infection with *B. henselae* results in a strong activation of HIF-1 whereas infection with *L. pneumophila* does not result in HIF-1 activation.
### Supplementary information, Tab. 1: Primers and PCR conditions

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References


