Endothelial cell mediators of angiogenesis in Bartonella henselae infection

Amy M. McCord

University of South Florida

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the American Studies Commons

Scholar Commons Citation
http://scholarcommons.usf.edu/etd/2620

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Endothelial Cell Mediators of Angiogenesis in *Bartonella henselae* Infection

by

Amy M. McCord

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine
Department of Medical Microbiology
College of Medicine
University of South Florida

Major Professor: Burt Anderson, Ph.D.
Thomas Klein, Ph.D.
Jonathan Harton, Ph.D.
Srikumar Chellappan, Ph.D.
Jane Koehler, M.D.

Date of Approval:
July 7, 2006

Keywords: cat scratch disease, bartonellosis, bacillary angiomatosis, apoptosis, chemokines, CXCL8, MCP-1

© Copyright 2006, Amy McCord
Chemokines – CXCL8..........................................................13

Ca$^{2+}$ Signaling...............................................................13

*B. henselae* and Angiogenesis........................................14

Objectives ........................................................................16

Materials and Methods....................................................18

Bacterial Strains..............................................................18

Cell Lines...........................................................................18

HMEC-1 Infections..........................................................19

Infections for CXCL8 ELISA..........................................20

CXCL8 ELISA.................................................................20

LPS and TLR Inhibition Assays ........................................21

Semiquantitative RT-PCR – mcp1.....................................22

RNA Extraction and Reverse Transcription....................23

Real-Time PCR...............................................................23

Semiquantitative RT-PCR – cxcr1,cxcr2..........................24

MCP-1 ELISA.................................................................25

Isolation of *B. henselae* LPS..........................................25

Enrichment of Outer Membrane Proteins from *B. henselae* and

Fractionation by Molecular Weight.................................25

HUVEC Proliferation Assay.............................................26

Chemotaxis of THP-1 Monocytes.................................27

*In vitro Capillary Tube Formation Assay*.......................28

Generation of *B. henselae* Secreted Proteins (BHSP)........28
Western Blotting ........................................................................................................29
Proliferation Assays with BHSP .............................................................................29
Measurement of CXCL8 Levels from BHSP-Treated HUVEC. ..................................30
Ca\(^{2+}\) Imaging ....................................................................................................30
Ca\(^{2+}\) Inhibitors ..................................................................................................31
Statistics ..................................................................................................................32

Results .......................................................................................................................33

Induction of MCP-1 Gene Expression and Protein Production ...............................33
Induction of MCP-1 Gene Expression and Protein Production is

Independent of B. henselae LPS and Endothelial Cell TLR4 .................................35

MCP-1 Production Requires NF\(\kappa\)B Activation ................................................37

Low Molecular Weight Outer Membrane Proteins (OMP-1) from

B. henselae Induce MCP-1 Production in HMEC-1 .................................................37

Supernatants from B. henselae-Infected HMEC-1 Induce

Chemotaxis of THP-1 Monocytes ........................................................................38

CXCL8 Production from a Variety of Cell Types Infected with

B. henselae ..............................................................................................................41

Effect of Blocking CXCL8 on B. henselae-Induced Endothelial

Cell Proliferation ..................................................................................................43

Role of CXCL8 in B. henselae-Induced Endothelial Cell Survival .........................46

Role of CXCL8 in B. henselae-Induced Capillary Tube Formation .........................48

B. henselae Secreted Proteins (BHSP) Contain GROEL and BadA .......................48

BHSP Induce a Proliferative Response in HUVEC .................................................51
BHSP Induce an Intracellular Ca\textsuperscript{2+} Response in HUVEC ........................................51
BHSP Induce an Intracellular Ca\textsuperscript{2+} Response in HUVEC from Intracellular Stores ..........................................................54
BHSP-Induced HUVEC Proliferation Requires Ca\textsuperscript{2+} ........................................54
BHSP Induce CXCL8 Production from HUVEC ..............................................................57
Discussion .......................................................................................................................59
Literature Cited ................................................................................................................71
Presentation of Studies .................................................................................................83
About the Author .........................................................................................................END PAGE
List of Figures

Figure 1. Comparison of parsimony tree (left side) and neighbor-joining tree (right side) derived from complete ITS sequences for recognized *Bartonella* species (type strains)...............................................................................................................2

Figure 2. Model of paracrine and autocrine mechanisms of *B. henselae*-mediated angiogenesis........................................................................................................................................7

Figure 3. Structure of TFSS apparatus of *B. henselae* .................................................................................................................................9

Figure 4. *B. henselae* stimulated MCP-1 induction in HMEC-1 .................................................................34

Figure 5. MCP-1 production during inhibition assays .................................................................................................36

Figure 6. MCP-1 production in response to OMP fraction 1 ...........................................................................................39

Figure 7. THP-1 monocyte chemotaxis in response to supernatants from *B. henselae*-infected HMEC .........................................................42

Figure 8. *B. henselae*-induced CXCL8 production assayed by ELISA ......................................................................................................44

Figure 9. CXCR2 expression in HUVEC .................................................................................................................................39

Figure 10. Effect of CXCL8 on HUVEC proliferation in response to *B. henselae* ..........45

Figure 11. Effect of anti-CXCL8 on inhibition of HUVEC apoptosis induced by *B. henselae* .................................................................47

Figure 12. Effect of anti-CXCL8 on *B. henselae*-induced capillary formation in a GFR matrigel .............................................................................49
Figure 13. *B. henselae* secreted proteins (BHSP) contain BadA and GROEL ..........50

Figure 14. BHSP cause HUVEC proliferation .................................................................52

Figure 15. BHSP cause a Ca^{2+} rise in HUVEC ..............................................................53

Figure 16. BHSP cause a Ca^{2+} rise in HUVEC from intracellular stores ....................55

Figure 17. Ca^{2+} signaling is important for BHSP-mediated HUVEC proliferation .......56

Figure 18. BHSP induce CXCL8 production from HUVEC .............................................58

Figure 19. Endothelial cell mediators of angiogenesis during *B. henselae* infection ......61
Endothelial Cell Mediators of Angiogenesis in *Bartonella henselae* Infection

Amy M. McCord

ABSTRACT

Bacillary angiomatosis (BA), one of the clinical manifestations resulting from infection with the facultative intracellular bacterium *Bartonella henselae*, is characterized by angiogenic lesions. Endothelial cells have been identified as host cells for this pathogen and are presumed important for pathogenesis as lesions contain bacteria in direct contact with the endothelium. Lesions also contain infiltrating macrophages, which contribute to the angiogenic process during *B. henselae* infection by secreting vascular endothelial growth factor (VEGF). While virulence factors have been characterized, and the role for macrophages in *B. henselae* infection has been established, endothelial cell mediators of angiogenesis have not been well defined. We investigated three potentially important pathways that are triggered by the bacterial interactions with endothelial cells. We examined the ability of endothelial cells to upregulate the chemokines monocyte-macrophage chemoattractant protein-1 (MCP-1) and CXCL8 and the mechanism by which *B. henselae* secreted proteins (BHSP) induce endothelial cell proliferation. We determined that MCP-1 production is upregulated in response to *B. henselae* infection, which very likely contributes to angiogenic lesion formation by recruiting the VEGF-secreting macrophage. The chemokine CXCL8 is an important mediator of angiogenesis which can cause endothelial cell survival, proliferation, and...
capillary tube formation. We determined that CXCL8 is secreted from *B. henselae*-infected cells and contributes to *B. henselae*-induced angiogenesis in an autocrine manner. We also investigated the role of Ca$^{2+}$ signaling during *B. henselae* infection. We determined that BHSP induce a robust intracellular Ca$^{2+}$ response in HUVEC which originates from intracellular Ca$^{2+}$ pools. Additionally, endothelial cell proliferation in response to BHSP required Ca$^{2+}$ activity, indicating a role for intracellular Ca$^{2+}$ pools during *B. henselae*-induced angiogenesis. Endothelial cell proliferation during *B. henselae* infection possibly indicates a mechanism by which a pathogen induces proliferation of its host cell in order to propagate its own survival. Numerous factors culminate in the vascular lesions that are characteristic of BA. *B. henselae* infection represents an important and unique model for pathogen-triggered angiogenesis, and studies into the specific mechanisms of this process may elucidate host cell-pathogen interactions as well as pathways of pathogenic angiogenesis.
Introduction

_Bartonella Species and Human Disease_

_Bartonella_ are short, pleomorphic, gram negative rods which are fastidious, aerobic, oxidase negative organisms (4). They are classified within the α₂ subgroup of the class proteobacteria. The single genus _Bartonella_ was created by merging _B. bacilliformis_ and the genus _Rochalimaea_ (13). There are currently 16 _Bartonella_ spp. identified. Figure 1 shows a parsimony tree for _Bartonella_ species derived from 16s rRNA sequences (37). _Bartonella_ spp. cause a chronic intraerythrocytic bacteremia in reservoir hosts and are transmitted by arthropod vectors during feeding. _Bartonella_ can cause an asymptomatic bacteremia. _B. quintana, B. bacilliformis, B. elizabethae, B. grahamii, B. vinsonii_ subsp. _berkhoffii, B. vinsonii_ subsp. _arupensis, B. henselae_, and _B. clarridgeiae_ cause human disease. _B. qunitana_ causes trench fever, a recurrent fever transmitted by human body lice, bacteremia, and bacillary angiomatosis (BA). _B. bacilliformis_ causes Carrion’s disease and Orroya fever with chronic verrugae. _B. henselae_ causes cat scratch disease (CSD) and BA. _B. quintana, B. henselae, B. elizabethae, B. vinsonii_ subsp. _berkhoffii, and B. vinsonii_ subsp. _arupensis_ have been associated with endocarditis, usually in patients previously diagnosed with valvulopathy (31).
Figure 1. Comparison of parsimony tree (left side) and neighbor-joining tree (right side) derived from complete ITS sequences for recognized Bartonella species (type strains). The support of each branch, as determined from 100 bootstrap samples, is indicated by the value at the node. The lengths of vertical lines are not significant. For the parsimony tree, the lengths of horizontal lines are also not significant. For the neighbor-joining tree, the scale bar represents evolutionary distance as calculated by using the Kimura two-parameter distance calculation. (37).
**Bartonella henselae**

*B. henselae* are gram-negative bacilli that display twitching motility. The genome size is around $1.9 \times 10^6$ bp (3). There are two phases of *B. henselae* in culture: the rough and smooth forms. The “rough” bacteria express a pilus-like structure that has been recently characterized as *Bartonella* adhesion A (BadA, see *Virulence factors*). The presence of pili correlates with more efficient attachment to host cells (4). The “rough” forms autoagglutinate, pit chocolate agar, and display a dry colony morphology (9). In contrast, the phase variation to “smooth” *B. henselae*, which correlates with increased passage number, is characterized by mucoid, non-pitting colonies, no auto-agglutination, and fewer pili (9).

**Diseases Caused by Bartonella henselae**

*B. henselae* is a flea-borne pathogen of cats and humans. Blood donors in the USA and Australia exhibit a 3-6% seroprevalence for *B. henselae* (12, 19, 29, 39, 47). Two manifestations of *B. henselae* infection include CSD and BA. CSD is a usually self-limiting lymphadenitis. While the lymph nodes usually regress after a period of weeks or months, in 10% of patients the lymphadenitis may become suppurative. Additionally, rash, hepatosplenomegaly, lytic bone lesions, and deep lymphadenitis may also occur. The vasoproliferative diseases BA and bacillary peliosis (BP) occur preferentially in immunocompromised patients. BA may occur with either *B. henselae* or *B. quintana* infections; however, BP is associated only with *B. henselae* (48). BA is a proliferative disorder of the vascular endothelium resulting in the formation of tumorous lesions on the skin (BA) and internal organs (BP) (86).
**B. henselae Pathogenesis**

*Bartonella spp.* are unique among bacteria for causing angioproliferation. Endothelial cells appear to be important during BA and BP as the lesions contain bacteria in direct contact with the endothelium. Histological examination of BA lesions has also revealed infiltration by polymorphonuclear leukocytes and macrophages. *B. henselae* pathogenesis can be divided into the effects of pro-inflammatory activation in the endothelial cell, autocrine promotion of proliferation in endothelial cells, inhibition of endothelial cell apoptosis, stimulation of endothelial cell proliferation, and paracrine effects from infection of macrophages and epithelial cells.

**B. henselae-Induced Pro-Inflammatory Activation**

NFκB is a target for pathogens that either promote or inhibit inflammation. NFκB activation in endothelial cells is characterized by surface expression of adhesion molecules such as E-selectin and intracellular adhesion molecule-1 (ICAM-1) and the release of Interleukin-8 (CXCL8, CXCL8). During *B. henselae* infection of endothelial cells, endothelial cell adhesion molecules are upregulated and NFκB is activated (32). ICAM-1 expression is also upregulated on HUVEC in response to infection (60). While many pathogens induce inflammation, only *Bartonella spp.* possess the ability to cause vasoproliferation. The role of the inflammatory response in *B. henselae* pathogenesis is not well understood; however, the bacteria may utilize this mechanism to attract key regulators of angiogenesis such as macrophages to the sites of infection, which secrete pro-angiogenic molecules and growth factors.
B. henselae-Induced Endothelial Cell Survival and Proliferation

Proliferation of endothelial cells is an important step during angiogenesis. B. henselae causes proliferation and migration of endothelial cells in vitro (20). B. henselae also causes enhanced survival of endothelial cells through inhibition of apoptosis. This mechanism consists of an inhibition of caspases 3 and 8 (43). Some aspects of B. henselae-induced endothelial cell proliferation are controversial and poorly defined. For example, some argue that direct contact with the bacterium is needed for proliferation, since the angiogenic factor of B. henselae was localized to the particulate fraction of the bacterium (20). Other studies revealed that direct contact is not needed for stimulation of endothelial cell proliferation, as the endothelial cells will proliferate if they are separated from the bacteria by a membrane (59). Another hypothesis is that the paracrine effect of macrophages is needed for proliferation. When supernatants from B. henselae-infected macrophages are added to endothelial cells, the cells proliferate at a higher rate than cells incubated with uninfected macrophage supernatants (71). These data revealed a role for the macrophage during infection as the macrophage produced potent endothelial growth factors in response to B. henselae infection. The effect of inhibition of apoptosis versus actual cell proliferation has also been debated (43, 77). These differences may be due to different passages of bacteria used, differing multiplicities of infection (MOIs), and different phases of the bacteria. Despite the presence of many conflicting ideas, B. henselae causes angiogenesis through the culmination of a multitude of factors.
**Paracrine Effectors During B. henselae–Induced Angiogenesis**

Peripheral cells are also key regulators of angiogenesis. During *B. henselae* infection, polymorphonuclear lymphocytes and macrophages are present in the angiogenic lesions from BA (49, 50, 64). Epithelial cells as well as macrophages produce VEGF in response to infection with *B. henselae* (41, 71, 77), which most likely also contributes to vascular proliferation during *B. henselae* infection. CXCL8 is also produced by *B. henselae*-infected epithelial cells (71, 77). These paracrine factors may also play a vital role in *B. henselae* induced endothelial cell proliferation and angiogenesis. A model of autocrine and paracrine effects on endothelial cells during *B. henselae* infection, which shows the relative contributions of peripheral and host cells in *B. henselae*-induced angiogenesis, is depicted in Figure 2 (71).

**B. henselae Virulence Factors**

The recent sequencing of the *B. henselae* genome has opened many new investigations into *B. henselae* virulence factors. While earlier investigations identified some putative virulence factors, such as outer membrane proteins (OMPs) and secreted factors, further identification and mutagenesis of new and important virulence factors has been facilitated recently. Recently identified or characterized virulence factors include the Type IV secretion system (TFSS), lipopolysaccharide (LPS), GROEL, and BadA.
**Figure 2.** Model of paracrine and autocrine mechanisms of *B. henselae*-mediated angiogenesis. *B. henselae* is able to adhere to and invade human macrophages (mac) and induce production of VEGF. This secreted VEGF functions in a paracrine manner and acts as an endothelial cell mitogens when it binds to its receptors on endothelial cells. Infection of endothelial cells (EC) with *B. henselae* may serve to further enhance proliferation by NFκB activation through upregulation of adhesion molecules such as E-selectin and ICAM-1. Also, chemokines may be produced upon endothelial cell infection which would attract macrophages leading to enhanced growth factor signaling. In addition, caspases are inhibited in infected endothelial cells leading to enhanced endothelial cell survival (Resto-Ruiz et.al., 2002).
TFSS

TFSS are multicomponent transport systems of gram-negative bacteria. They can mediate transfer of diverse factors, from effector proteins to DNA. The *B. henselae* TFSS is encoded by the *virB* operon. The *virB* operon is induced during endothelial cell infection (79). It has been recently determined that the TFSS encoded by the *virB* operon mediates invasion, proinflammatory activation, and anti-apoptotic protection of endothelial cells (77). However, at higher MOIs (larger than 50) the TFSS has a cytotoxic effect on the endothelial cell. Thus the proteins coded by *virB* are important for endothelial cell survival and invasion. This does not necessarily exclude the possibility of a factor which mediates proliferation. Recently a model of the TFSS apparatus was suggested which includes interactions between the VirB2 pilus-associated protein and VirB3 and VirB5 (Fig. 3) (81).

Recently, the proteins which are translocated by the TFSS machinery were identified as *Bartonella* effector proteins (Beps) A-G (80). Also, the VirD4 TFSS coupling protein was identified. BepD is translocated into HUVECs in a VirB/VirD4 dependent manner. The endothelial cell response to *B. henselae* with a deletion of the *virB4* includes a decrease in NFκB activation, endothelial cell survival, and bacterial invasion (77). *B. henselae* with deletions of *bepA-G* or *virD4* induced an endothelial response similar to that elicited from the endothelial cells infected with the *virB4* deletion mutant (80).
Figure 3. Structure of TFSS apparatus of \textit{B. henselae}. The proteins coded by the \textit{virB} operon of \textit{B. henselae} are thought to assemble as depicted here. VirB2 is thought to be the main conduit through which proteins are transported, which is driven by the VirB11 ATPase. VirB4 binds to itself as well as VirB10. VirB10 interacts with both VirB8 and VirB9. VirB5 and VirB3 exhibit the strongest interaction (Shamaei-Tousi et al., 2004).
Outer membrane proteins (OMPs)

The outer membrane of gram-negative bacteria serves as an interface between the host cell and the pathogen. *B. henselae* expresses a variety of outer membrane components, including lipopolysaccharide and a hemin-binding protein (HbpA), immunoreactive antigens, and a red blood cell invasion protein (IalB) (17). OMPs of 43-kDa and 66-kDa molecular masses bound HUVEC membrane components (14). *B. henselae* OMPs are important for pathogenesis (14, 32) and they have been implicated in an NFκB-dependent proinflammatory activation of endothelial cells (32). OMPs activate HUVEC dose-dependently as measured by E-selectin and ICAM-1 protein expression (32). They also bind HUVEC membrane proteins and may be important for bacterial adhesion and entry (14).

Bartonella Adhesin A (BadA)

Until recently, a putative Type IV pilus on the surface of *B. henselae* was presumed to exist (9). Transmission electron microscopy of *B. henselae* strains showed the presence of a pilus-like structure on the surface of the bacterium (9). This “pilus” mediates VEGF secretion from macrophages and host cell adhesion (41). The presence of the pili correlates with the “rough” phenotype of *B. henselae*. Recently, when the *B. henselae* genome was published (3) it was clear that the gene for the putative Type IV pilus was not present. Subsequently, the projections were determined to be homologous to the non-fimbrial adhesin *Yersinia* adhesion A (YadA) and were renamed BadA accordingly (72). Non-fimbrial adhesins are non-pillin structures which contain a connector domain, a fibrous stalk, and a C-terminus anchoring domain (69). BadA
mediates interaction of *B. henselae* with extracellular matrix proteins (ECM). In addition, BadA activates an important mediator of angiogenesis, hypoxia inducible factor (HIF-1), and elicits an anti-BadA immune response in mice and rabbits (72). Thus BadA is important for *B. henselae* infection and also may serve as a clinical marker for infection (72).

**Lipopolysaccharide (LPS)**

Although LPS is usually considered a pathogenic factor in Gram-negative bacteria, the LPS from *B. henselae* exhibits remarkably low endotoxicity. Bacteremic patients display no signs of septic shock. Unique components of the LPS structure include a pentaacyl Lipid A and a small inner core composed of an α-(2→4)-linked Kdo disaccharide with one glucose residue attached (97). In addition, *B. henselae* LPS does not signal through TLR4 or TLR2 (97). This low endotoxicity is consistent with other intracellular bacteria such as *Legionella* and *Chlamydia spp.*

**GroEL**

The heat shock response of *B. henselae* begins at temperatures of 37°C, human body temperature. The heat shock response includes production of GroEL, a 60 kDa protein; GroES, a 10 kDa protein; and DnaK, a 70 kDa chaperonin (35). GroEL from *B. bacilliformis* is mitogenic for HUVEC (63). *B. henselae* GroEL, although less potent, is also mitogenic for HUVEC (63). Antibodies to GroEL inhibit proliferation of HUVEC in response to *B. bacilliformis* lysate. GroEL was also present as a secreted protein (63).
Angiogenesis

Overview

Pathogenic (tumor or inflammatory) angiogenesis provides a survival mechanism for the tissues of tumors and tumor-like lesions (30). Angiogenesis is a multistep process during which the vessel wall disassembles, the basement membrane is degraded by matrix metalloproteinases (MMPs), endothelial cells migrate and invade the extracellular matrix, endothelial cells proliferate, and a capillary lumen is formed. Angiogenesis requires cooperation between cells, cytokines, growth factors, and matrix components. A sensitive balance between angiostatic and angiogenic factors must exist in order to control angiogenic activity; however in tumors and tumor-like lesions, this tightly regulated system is upset (36). Angiogenesis is associated with conditions that involve inflammatory cell infiltrate (34), such as cancer, papavavirus infection, and herpesvirus infection (7, 15, 28, 55, 61). Angiogenesis and inflammation coordinate through common stimuli for endothelial cells and leukocytes; these stimuli include chemokines. Angiogenic chemokines exert a direct effect on the endothelium and an indirect effect on angiogenic-factor expressing leukocytes (68, 88). Chemokines are induced by TNF or IL-1 or by interaction with bacterial pathogens and recruit leukocytes to sites of inflammation.
Chemokines – MCP-1

Monocyte-macrophage chemoattractant protein-1 (MCP-1) is a potent and specific monocyte agonist and chemoattractant (33, 96) which is produced by endothelial cells, smooth muscle cells, and macrophages in response to various stimuli, including LPS (93). MCP-1 stimulates chemotaxis of monocytes and macrophages to sites of inflammation (51). MCP-1 can also directly promote angiogenesis (68, 88). Thus MCP-1 could play dual roles in B. henselae-induced angiogenesis by acting in an autocrine manner on endothelial cells to promote angiogenesis, while recruiting macrophages, the effector cell in the model, to the site of infection.

Chemokines – CXCL8

CXCL8 is a member of a family of 8 structurally related chemokines that have been shown to induce angiogenesis. CXCL8 augments angiogenesis through enhanced endothelial cell survival, proliferation, and MMP production (52, 53). CXCL8 receptors CXCR1 and CXCR2 are widely expressed on normal and tumor cells (38, 84, 85, 95) and have been observed on endothelial cells (67, 74). These receptors also play a role in proliferation of endothelial cells (46).

Ca²⁺ Signaling

Calcium homeostasis may regulate important cellular functions including activation of signal transduction pathways, proliferation, invasion, and differentiation (26, 65, 75, 87). Ca²⁺ plays a key role during angiogenesis; however, the mechanisms involved are not fully explained (2). Depletion of intracellular pools of Ca²⁺ and not
cytosolic Ca\textsuperscript{2+} levels inhibits proliferation and migration of human vascular smooth
muscle cells (10, 83). In addition, inhibition of intracellular Ca\textsuperscript{2+} pools with thapsigargin
inhibits angiogenesis in the rat isolated aorta (82). Intracellular Ca\textsuperscript{2+} pools are crucial
mediators of angiogenesis.

**B. henselae and Angiogenesis**

*B. henselae*-induced angiogenesis is reminiscent of tumor angiogenesis. *B. henselae*-induced angiogenesis represents a paradigm for pathogen-triggered tumor formation (22). Recently, the steps for *B. henselae*-induced angiogenesis have been clarified to most likely include (i) an NF\kappa B-dependent proinflammatory activation, (ii) inhibition of endothelial cell apoptosis, (iii) direct endothelial cell proliferation, and (iv) growth factors produced from peripheral cells.

During angiogenesis, endothelial cells migrate and proliferate, then organize into vessels. As well as promoting endothelial cell proliferation, *B. henselae* causes angiogenesis *in vitro*. Kirby et. al. demonstrated that *B. henselae* promotes survival of endothelial cords and promotes invasion, survival, and differentiation in a collagen matrix (42). Thus in addition to inhibition of apoptosis and endothelial cell proliferation, *B. henselae* promotes capillary tube formation. Unfortunately, no small animal model of *B. henselae*-induced angiogenesis has been successfully developed. Zhang et. al. developed an animal model for *B. quintana* infection utilizing rhesus macaques (*Macaca mulatto*) (98). This model mimics the high-titer bacteremia in humans. A small animal model would allow for functional *in vivo* studies with bacterial mutants, further defining the roles of virulence factors in an *in vivo* environment.
*B. henselae*-induced angiogenesis represents a unique phenomenon in which a bacterium induces angiogenic lesions. One possible rationale for pathogen-induced angiogenesis during *Bartonella* infections is that the pathogen may improve its survival by propagation of its host cell. Vascularization during infection is exclusive for *Bartonella spp.* While many bacterial virulence factors have been characterized, the effects of these factors on the host cell are unknown. There are most likely numerous factors culminating in the vascular lesions that are a characteristic of BA. However, specific investigations into the direct effect of the bacterium on human endothelial cells may clarify pathogenic mechanisms of angiogenesis.
Objectives

*B. henselae* causes angiogenic lesions in the immunocompromised, a phenomenon unique to *Bartonella* spp. These lesions contain bacteria in direct contact with the endothelium as well as infiltrating macrophages and polymorphonuclear leukocytes. Macrophages secrete VEGF in response to *B. henselae* infection, thus they are thought to be quite important during infection. However, the bacteria induce endothelial cell proliferation in the absence of macrophages as well, so while VEGF signaling is probably important *in vivo*, there may also be direct stimulation of proliferation by the bacterium. Since the bacterial genome was published recently, there has been much progress in identification and characterization of virulence factors. However, the endothelial mechanisms contributing to angiogenesis during *B. henselae* infection have not been fully clarified. This study was developed to identify important endothelial cell mediators of angiogenesis during *B. henselae* infection and to identify the mechanisms involved in the upregulation of these factors. Investigations into endothelial cell contributions during *B. henselae*–induced angiogenesis may reveal the factor or factors responsible for angioproliferation. A review of the current literature led to the following hypothesis:

*B. henselae* causes upregulation of pro-angiogenic factors during infection of endothelial cells, which contributes to the overall pathogenesis of *B. henselae*. 
In order to refute or support this hypothesis, the following objectives were formulated:

1. To determine if *B. henselae* upregulates macrophage chemoattractant protein-1 production and expression in endothelial cells and the mechanism by which this occurs.

2. To determine the role of CXCL8 in *B. henselae*-induced endothelial cell proliferation, survival, and capillary tube formation.

3. To determine the effect of *B. henselae* secreted proteins on endothelial cell proliferation.
Materials and Methods

Bacterial Strains.

*Bartonella henselae* Houston-1 (ATCC 49882) (70) strain was grown on chocolate agar prepared with heart infusion agar base (Difco, Detroit, MI) supplemented with 1% bovine hemoglobin (Beckton Dickinson, Cockeysville, MD). Bacterial cultures were maintained at 37°C and 5% CO₂ and humidity to saturation. For certain experiments, bacteria were heat-killed at 100°C for 30 minutes as described previously (71). *Escherichia coli* JM109 strain was grown in Luria-Bertani broth or agar (Difco).

Cell Lines.

The immortalized human microvascular cell line (HMEC-1) (1) was cultured in MCDB131 cell culture media (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT), 10 ng/ml epidermal growth factor, 1.461 g/L L-glutamine, 1 μg/ml hydrocortisone, 50 μg/ml penicillin-streptomycin, 2.5 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO), 2 mg/ml sodium bicarbonate, and 10 mM HEPES (Mediatech, Herndon, VA). Human THP-1 monocytes (90) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS, 5 μM 2-mercaptoethanol (Sigma-Aldrich), 10 μg/ml vancomycin (Sigma-Aldrich), and 1 μg/ml amphotericin B. Human umbilical vein endothelial cells (HUVEC) were obtained from
Clonetics Corporation (San Diego, CA) and were cultured in EGM (Clonetics). HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in MEM containing 10% fetal calf serum, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 µM sodium pyruvate. Cells were maintained at 37°C and 5% CO₂ and humidity to saturation. HUVEC were used in experiments at passages from 4 to 7.

**HMEC-1 Infections.**

Prior to infection, the culture medium containing antibiotics was removed from cell cultures and replaced with media without antibiotics or growth factors. Cells were permitted to adapt overnight. *B. henselae* Houston-1 were harvested from chocolate agar and suspended in cell culture media, then concentration of bacteria was determined spectrophotometrically as described previously (44). Briefly, at OD₆₀₀, a reading of 0.5 correlates with 10⁹ colony forming units (cfu)/ml. *B. henselae* were added to cells at the multiplicity of infection (MOI) indicated. Cells were co-cultured with Houston-1 for various timepoints.

For *E. coli* co-cultures, JM109 were harvested into cell culture medium and added to cells at a density of 100 *E. coli* cells per HMEC-1, also determined spectrophotometrically. During the experiments with longer time courses and during inhibition assays, *E. coli* LPS (Sigma-Aldrich) was used as a positive control for MCP-1 production in order to keep HMEC-1 alive as *E. coli* JM109 infection was found to be cytotoxic for HMEC-1 at later time points.
Infections for CXCL8 ELISA.

To generate supernatants for the analysis of CXCL8 secretion, HUVEC, HepG2, HMEC-1, or THP-1 were placed into 24-well tissue culture plates (Costar, Cambridge, Mass.) at 90% confluency. THP-1 were differentiated by overnight incubation with 1 µM vitamin D3 (Sigma-Aldrich). Nonadherent cells were removed by washing. Cells were infected with the Houston-1 strain of *B. henselae* as described previously using the appropriate cell culture medium with no antibiotics (71). For the downstream analyses real-time PCR and capillary tube formation, the infections and incubations post-infection were carried out under serum-free conditions. Cells were infected at indicated MOIs.

CXCL8 ELISA.

To determine CXCL8 levels in supernatants from *B. henselae*-infected cells, the DuoSet ELISA development systems (R&D Systems, Minneapolis, Minn.) for human CXCL8 was used according to the manufacturer's directions. The 3,3',5,5'-tetramethylbenzidine Liquid Substrate System (Sigma-Aldrich) was added and left for 20 min. The horseradish peroxidase reaction was stopped with 2 N sulfuric acid. ELISA plates were analyzed using a µQuant plate reader (Bio-Tek, Winooski, VT.) at 450 nm.

HUVEC proliferation assay. HUVEC were seeded in 96-well plates at 1×10³ cells per well in media without antibiotics and allowed to adapt overnight. Cells were infected the following day with Houston-1 at an MOI of 50 or incubated with recombinant human CXCL8 (100 ng/ml, R&D Systems). Anti-human CXCL8 (10 µg/ml) or an isotype control (mouse IgG1, 10 µg/ml) were added to cell media during infections. After 72
hours, cells were fixed and examined with an inverted microscope and digital pictures were taken with a Kodak DC290. Cells were examined for qualitative differences in number. In addition, cells were counted in 5 high-powered fields per well and averaged.

**LPS and TLR Inhibition Assays.**

Polymyxin B sulfate (Sigma-Aldrich) was used to neutralize the bacterial LPS in some experiments. Houston-1 or *E. coli* JM109 or purified *E. coli* LPS was incubated with 30 µg/ml polymyxin B sulfate for 1 hour at 37°C and 5% CO₂ before adding to HMEC-1. In some experiments, HMEC-1 were preincubated with a mouse monoclonal antibody (HTA 125) specific for toll-like receptor-4 (TLR-4) (e-Bioscience, San Diego, CA) in order to determine if TLR-4 signaling was required for MCP-1 production. HMEC-1 were pre-incubated with 20 µg/ml anti-TLR4 for one hour at 37°C with gentle shaking, then co-cultures proceeded as usual and supernatants were collected eight hours after addition of bacteria or LPS. Live *E. coli* JM109 were cultured with HMEC-1 during some experiments in order to ensure that the TLR4 monoclonal antibody would block activity from the LPS of live *E. coli* as well as from purified LPS. An isotype control (mouse IgG2a, κ) (e-Bioscience) was used as a control antibody at 20 µg/ml. During NFκB inhibition assays, HMEC-1 were incubated with 50 µM pyrrolidinedithiocarbamate (PDTC) (Sigma) or 100 µM N-tosyl-L-phenylalanine (TPCK) (Sigma) for one hour at 37°C with 5% CO₂ before infections. Inhibitors were maintained throughout the course of the assays.
Semiquantitative RT-PCR – mcp1.

RT-PCR was performed on HMEC-1 co-cultured with B. henselae or E. coli at the indicated times after infection. Total RNA was extracted with Trizol reagent (Sigma-Aldrich) according to manufacturer’s protocol. Total RNA was treated with RNase-free DNase (Ambion, Inc., Austin, TX) according to the manufacturer’s instructions. Concentration of RNA was determined spectrophotometrically using a GeneQuant II (Pharmacia Biotech, Cambridge, England). cDNA preparation and subsequent PCR amplification were carried out by a One-Step RT-PCR kit (Qiagen, Inc., Valencia, CA) in the presence of gene-specific primers and 2 µg total RNA. The PCR conditions were 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C for 35 cycles. Primer sequences for RT-PCR were as follows: β-actin forward 5’-AGAAAATCTGGCACCACACC-3’; β-actin reverse: 5’-CCATCTCTTGCTCGAAGTCC-3’; MCP-1 forward 5’-TTCTCAAAACTGAAGCTCGCACTCTCGCC-3’; MCP-1 reverse: 5’-TGTGGAGTGAGTGTTCAAGTCTTGGGAGTT-3’. PCR products were analyzed by electrophoresis through 2% agarose gels and were visualized by ethidium bromide staining. Amplicon sizes were 434 bp and 348 bp for β-actin and MCP-1 primers, respectively. RT-PCR data were analyzed by scanning densitometry of gel bands with Kodak 1D Image Analysis software and normalizing to β-actin signals obtained from the same time points. RT-PCR reactions included a no template control and a no reverse transcriptase control to exclude DNA or RNA contamination.
RNA Extraction and Reverse Transcription.

Cells were infected at an MOI of 100 with Houston-1 or incubated with 100 ng/ml rCXCL8. 10 µg/ml anti-CXCL8 or an isotype control was added at the time of infection. After 24 hours, total RNA was extracted from HUVEC using TRIzol® reagent (Sigma-Aldrich) according to manufacturer’s protocol. Turbo DNA-free (Ambion, Austin, TX) was used to remove remaining DNA according to manufacturer’s protocol. Two micrograms of total RNA were transcribed with AMV reverse transcriptase (Promega, Madison, WI) and used for real-time PCR or semi-quantitative RT-PCR.

Real-Time PCR.

Primers used for real-time PCR were as follows: β-actin forward 5’ACCAACTGGGACGACATGGAGAAA3’, β-actin reverse 5’-TAGCACAGCCTGGATAGCAACGTA-3’; Bax forward 5’-TCTACTTTGCCAGCAACTGGTGTC-3’, Bax reverse 5’-TGTCACGCCCATGATGTCTGAT-3’; Bcl-2 forward 5’-ATTTCTGCA TCTCATGCCAGGG-3’, Bcl-2 reverse 5’-TGTCCTTTGCATTCTGAGGACGAGG-3’. β-actin was used as the housekeeping gene control. Real-time PCR was performed with a Bio-Rad iQ iCycler Detection System (Bio-Rad Laboratories, Ltd.) with iQSYBR Green supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Reactions were performed in a total volume of 25 µl with 400 nM concentrations of primers. Reactions consisted of 10 minutes at 95°C, 45 cycles of 15 s at 95°C, 15s at 58°C, and 30 s at 72°C. Melt curve analysis was used to determine PCR specificity. Melt curve analysis was run with 80 cycles of 10 s at 55°C with each cycle raising 0.5°C. All reactions were carried out in at
least duplicate for each sample. The standard curve method was used to determine amounts of each transcript. Relative expression of Bcl-2 or Bax was determined by dividing amount (ng) of Bax or Bcl-2 by amount of β-actin in each sample. Relative induction was determined by normalizing the relative expression of the uninfected control samples to 1. All experiments included no template controls and untranscribed (noRT) RNA controls.

**Semiquantitative RT-PCR – cxcr1, cxcr2.**

Reverse transcription-PCR (RT-PCR) was performed on HUVEC 24 hours after infection. Total RNA was extracted as described above. cDNA preparation and subsequent PCR amplification were carried out with a One-Step RT-PCR kit (QIAGEN, Inc., Valencia, CA) in the presence of gene-specific primers and 2 µg total RNA. The PCR conditions were 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C for 35 cycles. Primer sequences for RT-PCR were as follows: for β-actin forward, 5'-AGAAAA TCTGGCACCACACC-3'; for β-actin reverse, 5'-CCATCTCTTGCTCGAAGTCC-3'; for CXCR2 forward, 5'-ATTCTGGGATCCTTCACAG-3'; and for CXCR2 reverse, 5'-TGCACTTATTGCAGGAGGTCG-3'. PCR products were analyzed by electrophoresis through 2% agarose gels and were visualized by ethidium bromide staining. RT-PCR data were analyzed by scanning densitometry of gel bands with Kodak 1D Image Analysis software and normalizing to β-actin signals obtained from the same time points. RT-PCRs included a no-template control and a no-reverse-transcriptase control to exclude DNA or RNA contamination.
MCP-1 ELISA.

MCP-1 levels in infected and uninfected HMEC-1 supernatants were assayed by MCP-1 DuoSet ELISA development system (R & D Systems, Minneapolis, MN) according to manufacturer’s protocol. The 3, 3’, 5, 5’- tetramethylbenzidine liquid substrate system (Sigma-Aldrich) was added and the color was allowed to develop for 20 minutes. The reaction was stopped with 2N sulfuric acid. ELISA plates were analyzed using a μQuant platereader (Bio-Tek, Winooski, VT) at 450 nm.

Isolation of \textit{B. henselae} LPS.

\textit{B. henselae} LPS was isolated as described previously (92). Briefly, 3-day-old bacteria were harvested from chocolate agar and suspended in PBS. The bacterial pellet was washed three times with sterile water. Bacteria were lysed with lysis buffer (6% SDS, 60 mM Tris, 46% glycerol, 6% β-mercaptoethanol, 10mM dithiothreitol) at 100°C. Proteins were digested with proteinase K treatment. These crude extracts were concentrated by centricon YM-3 (Millipore Corp., Bedford, MA). Crude LPS was dialyzed against sterile endotoxin-free water for four days.

Enrichment of Outer Membrane Proteins from \textit{B. henselae} and Fractionation by Molecular Weight.

\textit{B. henselae} outer membrane proteins (OMPs) were enriched after inner membranes of total membrane preparations were solubilized with sarkosyl (14, 27). The sarkosyl-insoluble pellet was resuspended in 10 mM HEPES (pH 7.4). Protein
concentrations were determined using the Lowry protein assay (56). 600 µg protein in 400 µl Laemmli sample buffer with 142 mM 2-mercaptoethanol were heated at 95°C for 5 minutes and separated in a single large well of a 2-dimensional 4-12% Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA). Four sections of the gel were excised corresponding to 3-33kDa (OMP-1), 34-52 kDa (OMP-2), 53-97 kDa (OMP-3), and 98+ kDa (OMP-4). Each section was minced and proteins were eluted using the Model 422 Electro-Eluter (Bio-Rad, Hercules, CA) running at 30 milliamps for three hours in elution buffer consisting of 25 mM Tris base, 192 mM glycine, and 0.1% SDS. Buffer was then exchanged using Microcon-YM-3 filters (Millipore Corp). Protein concentrations were determined using the Lowry protein assay (56). During some experiments, the lower molecular weight fraction of OMPs (OMP-1) was treated with polymyxin B sulfate (30 µg/ml) or proteinase K. OMP-1 was added to proteinase K (10 µg/ml) and incubated at 55°C for 3 hours. Proteinase K was inactivated at 100°C for 15 minutes.

**HUVEC Proliferation Assay.**

HUVEC were seeded in 96-well plates at 1x10^3 cells per well in media without antibiotics and allowed to adapt overnight. Cells were infected the following day with Houston-1 at an MOI of 50 or incubated with recombinant human CXCL8 (100 ng/ml, R&D Systems). Anti-human CXCL8 (10 µg/ml) or an isotype control (mouse IgG1, 10 µg/ml) were added to cell media during infections. After 72 hours, cells were fixed and examined with an inverted microscope and digital pictures were taken with a Kodak
DC290. Cells were examined for qualitative differences in number. In addition, cells were counted in 5 high-powered fields per well and averaged.

Chemotaxis of THP-1 Monocytes.

Chemotaxis of THP-1 monocytes was examined with modified Boyden chambers (Neuroprobe, Cabin John, MD) according to manufacturer’s instructions. Briefly, the lower well of the chamber was filled with 1.2ml supernatants from uninfected, *B. henselae*-infected, or *E. coli*-infected HMEC-1. 510 µl of THP-1 cell suspension (5 x 10^5 cells) were added to the upper well. A 5 µm pore size PVP-free polycarbonate membrane (Neuroprobe) separated the two wells. Migration occurred while incubating the chambers at 37°C and 5% CO_2 with humidity for four hours. After four hours, the upper side of the membrane was scraped with a sterile swab soaked in PBS three times to remove non-migrated cells and the lower side of the membrane was fixed and stained with Hema-3 Stat Pack (Biochemical Sciences, Inc., Swedesbord, NJ) according to manufacturer’s instructions. Cells were counted in five high-powered fields per membrane and these numbers were averaged. Cell counts ranged from 6 to 10 cells per high-powered field, with the exception of cells migrated in response to uninfected HMEC-1 supernatants, in which case zero to two cells were counted per microscope field as a result of much less migration of THP-1 cells. Results are expressed as a chemotactic index (CI), in which the average number of cells that migrated in response to uninfected HMEC-1 supernatants was set to one. A graph of the means of CIs for three experiments is shown.
**In vitro Capillary Tube Formation Assay.**

A 96-well plate was coated with growth factor-reduced (GFR) Matrigel (BD Biosciences, Mountain View, CA). The matrigel contained no antibodies, a control isotype, or anti-CXCL8 (10µg/ml). The matrigel was solidified at 37°C for one hour, after which 10⁴ uninfected or *B. henselae*-infected (MOI=100) HUVEC were added to each well. rCXCL8 (100 ng/ml) was added to some wells containing uninfected cells at this time. After 18 hours, plates were examined for qualitative differences in capillary tube formation and photographs were taken with a Kodak DC290 digital camera.

**Generation of *B. henselae* Secreted Proteins (BHSP).**

*B. henselae* were harvested from chocolate agar plates and suspended in RPMI 1640. *B. henselae* were allowed to continue growth in flasks in RPMI 1640 for 24 hours at 220 rpm on an orbital shaker. After 24 hours of incubation, the suspension was removed from the flask and spun at 2000 rpm for 10 minutes to form a soft pellet. The supernatant was removed and passed through a 0.22 µm filtered to remove all bacteria. The bacteria-free supernatant was then concentrated in a Centricon-Plus 20 per manufacturer’s instructions (Millipore, Billerica, MA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL). Proteins were run on a 4-12% NuPAGE gel (Invitrogen) and visualized by silver staining (Bio-Rad). A vehicle control was also generated with identical methods, except that bacteria-free agar plates were swabbed and resuspended in RPMI 1640.
**Western Blotting.**

BHSP (10 µg) or vehicle control were run on NuPAGE gels and transferred to nitrocellulose membranes in the presence of NuPAGE transfer buffer (Invitrogen) according to manufacturer’s instructions. Alternatively, 10 µg BHSP or vehicle control were dotted on a nitrocellulose membrane. The membranes were blocked with TBST-5% skim milk overnight. The membranes were washed four times with TBST and then incubated with rabbit anti-BadA (1:1000 in blocking buffer) or rabbit anti-GROEL (1:500 in blocking buffer). The membranes were washed four times with TBST and incubated with a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:5000 in blocking buffer). The membranes were washed and bands or dots were detected using the ECL chemiluminescent substrate (Amersham Biosciences, Buckinghamshire, UK) and exposure to X-ray film.

**Proliferation Assays with BHSP.**

HUVEC were seeded in 96-well plates at a density of $10^3$ cells per well and allowed to attach overnight in media without antibiotics. BHSP or vehicle controls were added in EBM containing no antibiotics at indicated concentrations. After 72 hours, the HUVEC were photographed and 5 high-powered fields per well were counted and averaged.
Measurement of CXCL8 Levels from BHSP-Treated HUVEC.

HUVEC were treated with various concentrations of BHSP or medium controls. After 24 hours, the cell culture supernatants were collected and an ELISA was used to measure the CXCL8 levels as described under “CXCL8 ELISA”.

Ca^{2+} Imaging.

The calcium-sensitive dye fura2/AM was used for measuring intracellular free calcium concentrations in HUVEC, as previously described. Cells plated on coverslips were incubated for 1 h at room temperature in physiological saline solution (PSS) consisting of 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 7.7 mM glucose, and 10 mM HEPES (pH to 7.2 with NaOH), which also contained 1 μM fura-2/AM and 0.1% Me₂SO. The coverslips were then washed in PSS (fura-2/AM-free) prior to the experiments being carried out. PSS was applied via a rapid application system. *B. henselae* secreted proteins (BHSP) were applied with a pipette to the coverslip. Concentrated liquid culture media containing no bacteria served as a vehicle control.

A DG-4 high speed wavelength switcher (Sutter Instruments Co., Novato, CA) was used to apply alternating excitation with 340- and 380-nm UV light. Fluorescent emission at 510 nm was captured using a Sensicam digital CCD camera (Cooke Corp., Auburn Hills, MI) and recorded with Slidebook Version 3.0 software (Intelligent Imaging...
Innovations, Denver, CO) on a Pentium IV computer. Changes in \([\text{Ca}^{2+}]_i\) were calculated using the Slidebook 3 software (Intelligent Imaging Innovations, Denver, CO) from the intensity of the emitted fluorescence following excitation with 340- and 380-nm light, respectively, using the equation,

\[
[\text{Ca}^{2+}]_i = K_d Q (R - R_{\text{min}})/(R_{\text{max}} - R)
\]  

(Eq. 1)

where \(R\) represents the fluorescence intensity ratio \((F_{340}/F_{380})\) as determined during experiments, \(Q\) is the ratio of \(F_{\text{min}}\) to \(F_{\text{max}}\) at 380 nm, and \(K_d\) is the \(\text{Ca}^{2+}\) dissociation constant for fura-2. Calibration of the system was performed using a fura-2 calcium imaging calibration kit (Molecular Probes, Inc., Eugene, OR) and values were determined to be as follows: \(F_{\text{min}}/F_{\text{max}} = 23.04; R_{\text{min}} = 0.31; R_{\text{max}} = 8.87\).

**Ca^{2+} Inhibitors.**

The intracellular \(\text{Ca}^{2+}\) inhibitor thapsigargin was used in some assays when indicated. Cells were incubated for 30 minutes with 10 µM thapsigargin followed by 30 minute incubation with Fura2/AM. In some experiments, \(\text{Ca}^{2+}\) imaging was performed in the absence of extracellular \(\text{Ca}^{2+}\) by using PSS without \(\text{Ca}^{2+}\). BAPTA/AM, a \(\text{Ca}^{2+}\) chelator, was used during some experiments to inhibit \(\text{Ca}^{2+}\) signaling. The cells were
pre-incubated with various concentrations of BAPTA/AM or DMSO for 10 minutes at 37°C and then washed to remove excess BAPTA/AM or DMSO.

**Statistics.**

Significance was determined by a Student’s t test with two-tailed distribution. P values less than 0.05 were considered significant. Statistical significance of Ca\(^{2+}\) imaging data was determined by two-way ANOVA. All experiments were performed in triplicate. Data are presented as a mean ± one SD.
Results

Induction of MCP-1 Gene Expression and Protein Production.

During *B. henselae* infection, monocytes and macrophages infiltrate the angiogenic lesions (49, 50, 64). MCP-1 is a C-C chemokine which recruits monocytes and macrophages to sites of infection through binding to its CCR2B receptor (16). We investigated the effect of *B. henselae* infection on MCP-1 expression and production in HMEC-1. MCP-1 transcript levels were assayed by semi-quantitative RT-PCR at 6 and 24 hours after infection (Fig. 4). *B. henselae*-infected HMEC-1 had 1.6 times higher levels of MCP-1 transcript than uninfected controls at 6 hours after infection (T6) and 1.8 times higher levels of MCP-1 transcript 24 hours after infection (T24) (Fig. 4A, B). By 48 hours after infection, the MCP-1 message levels for uninfected HMEC-1 began to approach the MCP-1 message levels of *B. henselae*-infected HMEC-1 (data not shown). This was most likely due to the absence of growth factors in culture media; after 48 hours of culture with no growth factors, cells start to die and MCP-1 levels increase.

MCP-1 levels in supernatants of infected or uninfected HMEC-1 were analyzed at 6, 24, and 48 hours after infection by ELISA (Fig. 4C). There was a significant increase in MCP-1 levels when HMEC-1 were infected with *B. henselae* for 6, 24, or 48 hours (P<0.007). In order to determine whether the bacterial stimulating molecule was heat stable, bacteria were heat-killed by boiling a 100°C for 15 minutes before addition to
Figure 4. *B. henselae* stimulated MCP-1 induction in HMEC-1. (A) Cells were stimulated with *B. henselae* or *E. coli*. Some bacteria were preincubated with polymyxin B sulfate. The levels of MCP-1 mRNA in HMEC-1 were assayed by RT-PCR at 6 and 24 hours after infection (T6, T24). (B) RNA levels of MCP-1 were determined by scanning densitometry and normalized by comparison to β-actin RNA levels (C) MCP-1 protein production was determined by ELISA at 6, 24, and 48 hours after infection. (UN=uninfected cells; BH=*B. henselae*-infected cells; PB=polymyxin B sulfate; EC=*E. coli* infected cells; *P<0.007, *B. henselae* infection compared to uninfected cells; **P<0.007, untreated *E. coli* compared to *E. coli* treated with polymyxin B sulfate). Results shown are one representative of three similar experiments.
HMEC-1. When bacteria were heat-killed, MCP-1 levels were not significantly lowered (data not shown). These data indicate that a heat-stable molecule is responsible for MCP-1 production.

**Induction of MCP-1 Gene Expression and Protein Production is Independent of *B. henselae* LPS and Endothelial Cell TLR4.**

The cell walls of Gram-negative bacteria contain LPS, which instigates a pro-inflammatory response from cells through signaling through TLR4. This signaling may lead to upregulation of pro-inflammatory cytokines such as IL-1β, TNF-α, and MCP-1. We investigated the role of *B. henselae* LPS in MCP-1 production from HMEC-1. Bacteria (*B. henselae* or *E. coli*) were treated with polymyxin B sulfate before addition to HMEC-1. Although incubation of *E. coli* with polymyxin B sulfate significantly lowered MCP-1 gene expression and protein production from HMEC-1 at T6, T24, and T48 (P ≤ 0.006), polymyxin B had little effect on *B. henselae*-induced MCP-1 expression and production (Fig. 4A, B, C).

MCP-1 production can be mediated by TLR4 or TLR2 activation (89). Since HMEC-1 express TLR2 very weakly (25), we investigated whether *B. henselae*-induced MCP-1 production is mediated through TLR4 on HMEC-1. We preincubated HMEC-1 with a TLR4 mouse monoclonal antibody (HTA125) or an isotype control (mouse IgG2a, κ), then infected with *B. henselae* or added *E. coli* LPS (100 ng/ml). Supernatants were collected 8 hours after infection and assayed for MCP-1 by ELISA. While HTA125 reduced MCP-1 production caused by *E. coli* LPS (P < 0.002), *B. henselae*-induced MCP-1 production remained unchanged by the blocking antibody (P > 0.900) (Fig. 5A). These
Figure 5. MCP-1 production during inhibition assays. Supernatants were collected 8 hours after infection and analyzed by ELISA for MCP-1 levels. (A) Cells were incubated with anti-tlr4 (HTA125) or a control isotype (mouse IgG2a, κ) for 1 hour before infection with *B. henselae* or addition of *E. coli* LPS (100 ng/ml). (B) HMEC-1 were cultured in the presence of the NFκB inhibitors PDTC or TPCK before infection with *B. henselae* or addition of *E. coli* LPS (1 µg/ml). (UN=uninfected HMEC-1; BH=*B. henselae*-infected HMEC-1; EC=HMEC-1 stimulated with *E. coli* LPS; *P<0.002; **P<2.00 X 10^-4*). A representative of three experiments is shown.
data indicate that the MCP-1 produced in response to *B. henselae* from HUVEC does not involve endothelial cell TLR4 or *B. henselae* LPS.

**MCP-1 Production Requires NFκB Activation.**

The *mcp1* gene has binding sites for the transcription factors NFκB and AP-1 (73). It was established that *B. henselae* can induce NFκB activation in endothelial cells (32). In order to determine if the transcription factor NFκB was responsible for MCP-1 production in HMEC-1 infected with *B. henselae*, NFκB inhibitors PDTC and TPCK were added to cells before infection. MCP-1 production in response to both *B. henselae* infection and *E. coli* LPS (1µg/ml) decreased significantly in the presence of either inhibitor (P<0.0002) (Fig. 5B). Induction of MCP-1 production by *B. henselae* is NFκB dependent.

**Low Molecular Weight Outer Membrane Proteins (OMP-1) from *B. henselae***

**Induce MCP-1 Production in HMEC-1.**

Since MCP-1 production in response to *B. henselae* infection occurs in an LPS-independent manner, we investigated whether OMPs of the bacteria stimulate MCP-1 production in HMEC-1. It has been shown that OMPs induce NFκB activation in human umbilical cord vein endothelial cells (HUVEC) (32). Because NFκB is one of the transcription factors that induces MCP-1 gene expression (73, 91), we investigated whether OMPs could induce production of MCP-1 from HMEC-1. Outer membrane proteins were enriched from *B. henselae* as previously described (14). The OMPs were separated into four molecular weight fractions (see Materials and Methods), and OMPs
were added to HMEC-1 culture at 250 ng/ml. Out of four fractions, only the lowest molecular weight fraction (from 3-33 kDa), Fraction 1 (OMP-1), significantly increased MCP-1 production in HMEC-1 at 6 and 24 hours after addition of OMPs (P<0.002) (Fig. 6A). OMP-1 also caused MCP-1 production from HMEC-1 in a dose-dependent manner (Fig. 6A). Preincubation of OMP-1 with Polymyxin B sulfate did not abrogate its effect (P>0.700) (Fig. 6B), suggesting that endotoxin contamination in the OMP-1 preparation is not responsible for MCP-1 induction. However, proteinase K treatment significantly lowered the effect of OMP-1 (P<0.0002), revealing that the factor in OMP-1 which upregulates MCP-1 production is probably a protein or proteins.

**Supernatants from B. henselae-Infected HMEC-1 Induce Chemotaxis of THP-1 Monocytes.**

Since MCP-1 levels are heightened in response to B. henselae infection, we investigated whether supernatants from B. henselae-infected HMEC-1 could cause chemotactic migration of THP-1 monocytes. A modified Boyden chamber assay was used to assess the chemotactic response of THP-1 monocytes to chemoattractants present in the supernatant from B. henselae-infected HMEC-1. Migrated cells were fixed, stained, and counted in 5 high-powered fields. Supernatants from uninfected HMEC-1 were used as negative controls and supernatants from E. coli-infected HMEC-1 were used as positive controls. Supernatants from B. henselae-infected HMEC-1 induced THP-1 chemotaxis at a level about 6 times that of supernatants from uninfected HMEC-1 (Fig. 7) and at similar levels to supernatants from E. coli-infected HMEC-1 (4 times that of uninfected supernatants). These results indicate that the levels of MCP-1 and/or other
Figure 6. MCP-1 production in response to OMP fraction 1. HMEC-1 were stimulated with OMP-1 under various dosages and conditions. Supernatants were collected at 6 and 24 (T6, T24) hours after addition of OMP-1. MCP-1 levels were determined by ELISA. (A) Dose-dependent response of HMEC-1 to various OMP-1 concentrations (B) Effects of polymyxin B sulfate (30 µg/ml) (PB) and proteinase K (10 µg/ml) (PK) on OMP-1 induced MCP-1 production in HMEC-1. (*P<0.002; **P<2.00 X 10^{-4}). A representative of three experiments is shown.
Figure 7. THP-1 monocyte chemotaxis in response to supernatants from *B. henselae*-infected HMEC. (A) Chemotaxis of THP-1 monocytes in response to uninfected HMEC supernatants, *B. henselae*-infected HMEC supernatants (BH), or *E. coli*-infected HMEC supernatants (EC). Results are expressed as a chemotactic index. The number of migrated THP-1 in five microscopic fields was averaged for supernatants from uninfected, *B. henselae*-infected, and *E. coli*-infected HMEC-1. The chemotactic index for cells which migrated in response to uninfected supernatants was set at one, and other samples were normalized accordingly. Results shown are the mean of three separate experiments.
chemokines in supernatants from infected HMEC-1 are sufficient to cause chemotaxis of monocytes.

**CXCL8 Production from a Variety of Cell Types Infected with B. henselae.**

CXCL8 plays a role in angiogenesis through induction of MMP production, endothelial cell survival, and capillary tubule formation (52). It has been reported that CXCL8 production is enhanced from endothelial cells and epithelial cells by an NFκB-dependent pathway in the presence of *B. henselae* (32, 77). We tested a variety of cell types for production of CXCL8 in response to *B. henselae* at an MOI of 100. Two types of endothelial cells, HMEC-1 and HUVEC (see Materials and Methods), upregulated CXCL8 in response to *B. henselae* 24 hours after infection (Fig. 8A). Other cells which may be important for *B. henselae* pathogenesis (hepatocytes and monocyte-derived macrophages) were also examined for their ability to upregulate CXCL8 in response to *B. henselae*. They also markedly upregulated CXCL8 during infection (Fig. 8B, C). We have previously reported (71) that *B. henselae*-infected THP-1 do not enhance CXCL8 production at an MOI=500, which conflicts with the results presented here. However, this study used Vitamin D3 for monocyte differentiation (see Materials and Methods) in place of phorbol myristate acetate (PMA), which lowers background CXCL8 production; thus the differences between uninfected and infected macrophages were more clearly distinguishable.

Furthermore, we examined HUVEC for CXCL8 receptors CXCR1 and CXCR2 expression in the presence of *B. henselae*. While CXCR1 RNA levels were not significantly different between uninfected and infected HUVEC (data not shown),
Figure 8. *B. henselae*-induced CXCL8 production assayed by ELISA. (A) *B. henselae* induced CXCL8 production from human umbilical vein endothelial cells (HUVEC) and; human microvascular endothelial cells (HMEC-1). (B) *B. henselae*-induced CXCL8 production from hepatocytes (HepG2). (C) *B. henselae*-induced CXCL8 production from monocyte-derived macrophages (THP-1). (Un=Uninfected cells; Bh=B. henselae-infected cells; *P<0.001).
CXCR2 levels were around four times higher in *B. henselae*-infected cells than in uninfected cells (Fig. 9A, B). *B. henselae* causes CXCL8 production from macrophages, endothelial cells, and hepatocytes; *B. henselae* also induces CXCL2 expression in endothelial cells, indicating a putative autocrine mechanism during *B. henselae* infection of endothelial cells.

**Effect of Blocking CXCL8 on *B. henselae*-Induced Endothelial Cell Proliferation.**

*B. henselae* causes more endothelial cell proliferation at MOIs of under 50 than at higher MOIs (77). This is most likely due to a cytotoxic effect from a factor coded by the virB operon at higher MOIs. However, other aspects of *B. henselae*-induced angiogenesis such as inhibition of apoptosis, capillary tube formation, and NFκB-dependent proinflammatory activation correlate positively with bacterial numbers (42, 43, 72). Since it has been reported that CXCL8 directly mediates endothelial cell survival and proliferation (46), we examined the role of CXCL8 in *B. henselae*-induced HUVEC proliferation (Fig. 10A). Cells were incubated with *B. henselae* (MOI of 50) or rCXCL8 (100 ng/ml). Cells were also treated with anti-human CXCL8 or a control IgG1 (10 µg/ml). After 3 days, pictures were taken of the wells (Fig. 9A) and cells were counted (Fig. 10B). Both *B. henselae* and rCXCL8 induced proliferation as compared to unstimulated cells. The presence of a CXCL8 antibody quenched the proliferative effect of *B. henselae* and rCXCL8, while an isotype control did not. These data point to a putative role for CXCL8 during *B. henselae*-induced proliferation.
**Figure 9. CXCR2 expression in HUVEC.** (A) RNA was extracted from uninfected and *B. henselae*-infected HUVEC and subjected to semi-quantitative RT-PCR. (B) Scanning densitometry determined the relative intensities of CXCR2 expression when normalized to β-actin house-keeping gene expression. Relative CXCR2 induction was measured as a ratio of CXCR2 to β-actin when this ratio in uninfected cells was normalized to 1. *(UN=uninfected HUVEC; BH= B. henselae-infected HUVEC).*
Figure 10. Effect of CXCL8 on HUVEC proliferation in response to *B. henselae*. HUVEC were uninfected (UN) or infected with *B. henselae* (BH) or incubated with rCXCL8 in either the presence or absence of a neutralizing anti-CXCL8 antibody or an isotype control (mouse IgG1) for 3 days. (A) Digital pictures of typical phase-contrast microscopic fields. (B) Cell proliferation expressed as a graph of average cell number per high-powered field. *P*<0.008.
Role of CXCL8 in *B. henselae*-Induced Endothelial Cell Survival.

The balance between Bax (apoptotic) and Bcl-2 (anti-apoptotic) is important for endothelial cell survival or apoptosis. We examined HUVEC infected with *B. henselae* at an MOI of 100 for expression of Bcl-2 family members Bcl-2 and Bax. CXCL8 induces increased Bcl-2 expression and decreased Bax expression (53). While it has been reported that *B. henselae* inhibits apoptosis of HUVEC though inhibition of caspases (43), the Bcl-2 and Bax levels in uninfected and *B. henselae*-infected HUVEC have not been previously compared. We examined Bax and Bcl-2 levels in HUVEC by real-time PCR. We found that *B. henselae*-infected HUVEC had almost undetectable levels of Bax expression and about four times enhanced Bcl-2 expression (Fig. 11A) when compared with uninfected controls and normalized to a β-actin housekeeping gene. This increased Bcl-2/Bax ratio probably biases the cell into an anti-apoptotic state. We examined the role of CXCL8 on *B. henselae*-enhanced HUVEC survival. When anti-CXCL8 was added to HUVEC in the presence of *B. henselae*, the anti-apoptotic response of the cells decreased markedly (Fig. 11B, C). Bax levels were raised about fivefold in the presence of anti-CXCL8 but not in the presence of control IgG1 (Fig. 11B). Conversely, Bcl-2 levels induced by *B. henselae* infection dropped six fold in the presence of a CXCL8 neutralizing antibody (Fig. 11C). These results reveal a possible autocrine role for CXCL8 in *B. henselae*-stimulated endothelial cell survival.
Figure 11. Effect of anti-CXCL8 on inhibition of HUVEC apoptosis induced by *B. henselae*. (A) *B. henselae* causes enhanced Bcl-2 expression and decreased Bax expression in HUVEC. Results are expressed as relative expression units, a ratio of amounts of Bcl-2 or Bax transcripts to β-actin transcript amounts. (B) Bax expression reduced by *B. henselae* is increased in the presence of a neutralizing antibody to CXCL8. (C) Bcl-2 expression increased by *B. henselae* is decreased in the presence of anti-CXCL8. (UN=uninfected HUVEC; BH=B. henselae–infected HUVEC; rCXCL=rCXCL8-treated HUVEC; *P<0.01).
**Role of CXCL8 in *B. henselae*-Induced Capillary Tube Formation.**

*In vitro* angiogenesis assays have revealed a pro-angiogenic response of HUVEC to *B. henselae* infection (42). HUVEC infected with *B. henselae* seeded on a GFR matrigel exhibited advanced capillary tube formation when compared to uninfected HUVEC (Fig. 12). HUVEC incubated with rCXCL8 also showed enhanced capillary tube formation. When anti-CXCL8 was present in the matrigel, the capillary tube formation was visibly diminished (Fig. 12). The presence of an isotype control, however, had no such effect on tube formation. These data delineate further an autocrine role for CXCL8 during *B. henselae* infection.

**B. henselae Secreted Proteins (BHSP) Contain GROEL and BadA.**

*B. henselae* secreted proteins have been implicated in endothelial cell proliferation (59). However, the TFSS encoded by the *virB* operon is not responsible for endothelial cell proliferation, and is turned on only inside the endothelial cell (77, 79). Thus in order to avoid this cytotoxic effect and study simply the effect of TFSS-independent secreted proteins, we isolated secreted proteins (SP) from *B. henselae* conditioned media as described in *Materials and Methods*. The proteins were analyzed by electrophoresis and tested for the presence of GROEL, a heat shock protein that is potentially secreted, and BadA, an immunogenic adhesion (Fig. 13A, B), by western blot and dot blot, respectively. Both proteins were present in the SP fractions. The presence of GROEL was expected, as it has been previously found in conditioned media from *B.
Figure 12. Effect of anti-CXCL8 on *B. henselae*-induced capillary formation in a GFR matrigel. HUVEC were infected with *B. henselae* (BH) or uninfected (UN) and seeded on a GFR matrigel containing no antibody, a control antibody (mouse IgG1, 10 µg/ml), or anti-CXCL8 (10 µg/ml). Uninfected HUVEC were then either stimulated with CXCL8 (100 ng/ml) or left alone. Pictures were taken after 24 hours.
Figure 13. *B. henselae* secreted proteins (BHSP) contain BadA and GROEL.

BHSP were analyzed by western blot for GROEL (A) and dot blot for BadA (B). A medium control (MC) served to rule out non-specific antibody binding to proteins in the medium. Secreted proteins from a BadA mutant (ΔBadA) were also used as a negative control for the BadA dot blot.
henselae (63). However, the presence of BadA was unexpected since it is an adhesin; perhaps it is shed from the bacteria during liquid culture. Thus we identified two potentially important proteins in the B. henselae secreted protein (BHSP) fraction.

**BHSP Induce a Proliferative Response in HUVEC.**

There has been controversy in the literature concerning whether bacteria-host cell contact is needed for endothelial cell proliferation to occur (20, 59). Therefore we tested the ability of BHSP to cause proliferation in HUVEC. At a concentration of 250 µg/ml, the BHSP caused HUVEC proliferation, while a medium control (MC) at the same concentration did not (Fig. 14A, B). The proliferative response was almost 3 times that of untreated controls. In addition, the morphology of the cells incubated with BHSP was similar to the morphology of HUVEC when they are infected with B. henselae at MOIs of 50 or lower. They are elongated and display morphology consistent with proliferating cells in the presence of VEGF (77).

**BHSP Induce an Intracellular Ca\textsuperscript{2+} Response in HUVEC.**

Ca\textsuperscript{2+} signals play a key role in angiogenesis and other cellular processes (2). We tested the ability of BHSP to induce a Ca\textsuperscript{2+} rise in HUVEC. Interestingly, when 250 µg BHSP were added to HUVEC, intracellular Ca\textsuperscript{2+} concentrations rose rapidly to 100 nM (Fig. 15A). When a medium control was added to HUVEC, there was no Ca\textsuperscript{2+} rise (Fig. 15A). The peak Ca\textsuperscript{2+} concentrations were significantly different (P<0.05) from base levels (Fig. 15B).
Figure 14. BHSP cause HUVEC proliferation. HUVEC were incubated with a medium control (MC) or BHSP (250 µg/ml) and incubated 96 hours. Cells were viewed by inverted microscope and pictures were taken to view qualitative cell numbers (A). Five fields per well were counted and the average number for media controls was normalized to 1 (B).
Figure 15. BHSP cause a Ca\(^{2+}\) rise in HUVEC. BHSP were added to HUVEC mounted on a coverslip and intracellular Ca\(^{2+}\) was quantified as described in Materials and Methods. (A) One HUVEC Ca\(^{2+}\) response to BHSP (250 µg/ml) and a medium control (MC). (B) The average peak and baseline Ca\(^{2+}\) levels were compared for all cells. (*P<0.05; results are expressed as the mean plus one standard deviation).
BHSP Induce an Intracellular Ca\(^{2+}\) Response in HUVEC from Intracellular Stores.

In order to determine if the intracellular Ca\(^{2+}\) response from HUVEC resulted from extracellular Ca\(^{2+}\) entering the cell or from Ca\(^{2+}\) mobilization from intracellular stores, we tested the Ca\(^{2+}\) response to BHSP under various conditions. Under extracellular Ca\(^{2+}\)-free conditions (0 Ca\(^{2+}\)), the Ca\(^{2+}\) response did not change significantly (Fig. 16A, B) upon application of BHSP. Thus the Ca\(^{2+}\) response derived from inside the cell. Next we preincubated HUVEC with 1 µM thapsigargin (THAPS), a Ca\(^{2+}\) ATPase inhibitor which depletes intracellular Ca\(^{2+}\) stores (10, 83). In the presence of the inhibitor, the Ca\(^{2+}\) levels did not increase in response to BHSP (Fig. 15A) and the peak values of Ca\(^{2+}\) concentration were significantly lowered (Fig. 16B, P<0.05). Therefore the BHSP-derived Ca\(^{2+}\) response in HUVEC derives from intracellular Ca\(^{2+}\) pools, which have been implicated as the crucial Ca\(^{2+}\) pool for angiogenesis (10, 80, 83).

BHSP-Induced HUVEC Proliferation Requires Ca\(^{2+}\).

Given that intracellular Ca\(^{2+}\) is important for angiogenesis and that BHSP induce such a high concentration of intracellular Ca\(^{2+}\) as well as HUVEC proliferation, we investigated whether HUVEC proliferation induced by BHSP would still occur in the presence of a Ca\(^{2+}\) chelator, BAPTA/AM. After 10 minute pre-incubation with BAPTA/AM at concentrations of 1 µM BAPTA/AM or equivalent volumes of a DMSO vehicle control, the cells were washed and BHSP or medium controls were added. In the presence of BAPTA/AM, the HUVEC proliferation was reduced almost 50% (Fig. 17). We also determined that whole \textit{B. henselae}-induced proliferation is also lowered when
Figure 16. BHSP cause a Ca$^{2+}$ rise in HUVEC from intracellular stores.

HUVEC were either incubated with 1 µM thapsigargin (THAP) for 30 minutes followed by incubation with FURA-2-AM (20 µM), or incubated with FURA-2-AM and assayed in Ca$^{2+}$-free conditions. BHSP were added to HUVEC and intracellular Ca$^{2+}$ was quantified as described in Materials and Methods. (A) Responses to BHSP from cells assayed with Ca$^{2+}$ present (control), cells assayed in the absence of Ca$^{2+}$ (0 Ca), and cells incubated with 1 µM thapsigargin (THAPS). (B) The average peak and baseline Ca$^{2+}$ levels were compared for all cells. Results are expressed as the mean plus one standard deviation. (*P<0.05).
Figure 17. \(\text{Ca}^{2+}\) signaling is important for BHSP-mediated HUVEC proliferation. HUVEC were preincubated with 1 µM BAPTA/AM or a DMSO vehicle control. A medium control (MC) or BHSP were added to HUVEC at indicated concentrations 250 µg/ml, or cells were infected at an MOI of 50 with \(B.\) henselae (BH). After 72 hours, cells were photographed and five high-powered fields (HPF) were counted and averaged (*P<0.02; **P<0.04).
HUVEC are pre-incubated with BAPTA/AM. Consequently, intracellular Ca\(^{2+}\) is important for BHSP- and live *B. henselae*-induced HUVEC proliferation.

**BHSP Induce CXCL8 Production from HUVEC.**

We have determined that CXCL8 plays an autocrine role in *B. henselae*–induced endothelial cell survival, proliferation, and capillary tube formation. In order to determine if the BHSP were inducing proliferation through CXCL8 production, we tested the ability of BHSP to induce CXCL8 production from HUVEC. When BHSP were added to HUVEC, the CXCL8 levels increased (Fig. 18A). CXCL8 production did not increase in the presence of a medium control. Additionally, when BAPTA/AM was added to HUVEC before addition of BHSP, the CXCL8 levels dropped significantly (Fig. 18B). These data indicate a role for intracellular Ca\(^{2+}\) activity in CXCL8 production mediated by BHSP.
Figure 18. BHSP induce CXCL8 production from HUVEC. (A) HUVEC were incubated with a medium control (MC) or BHSP at indicated concentrations (µg/ml) for 24 hours. Supernatants were collected and ELISA was performed. (B) HUVEC were preincubated with BAPTA/AM or DMSO control. BHSP or media controls were added at shown concentrations (µg/ml) and supernatants were collected after 24 hours and ELISA was performed. *P<0.03.
Discussion

*B. henselae*, the etiologic agent of CSD, is a fastidious, gram-negative, oxidase-negative, aerobic bacillus (11, 13). *B. henselae* infections cause a range of symptoms from lymphadenopathy (CSD) to systemic disease. The severity of the disease tends to relate to immune status. Immunocompromised patients such as AIDS patients, chronic alcoholics, or immunosuppressed people can develop systemic disease. However, immunocompetent patients may still present with systemic bacteremia, endocarditis, and bacillary angiomatosis.

*B. henselae* can cause vascular proliferative lesions (5) into which macrophages infiltrate during infection (49, 50, 64). In the paracrine and autocrine model of *B. henselae*-induced angiogenesis (Fig. 2), macrophages are implicated as effector cells; upon stimulation by *B. henselae*, they secrete VEGF and other endothelial cell mitogens (71). Concurrently, endothelial cells upregulate pro-angiogenic factors such as chemokines, inhibit apoptosis through inhibition of caspases (44), and upregulate adhesion molecules (32) which may promote proliferation. In this study we investigated the endothelial cell mediators of angiogenesis which are induced upon infection with *B. henselae*. Specifically, we determined that *B. henselae* (i) upregulates MCP-1 production, which brings the effector cell macrophage into the site of infection, where it secretes VEGF and CXCL8 which would promote angiogenesis; (ii) induces CXCL8
production and CXCL8 receptor CXCR2 expression, which promotes angiogenesis in an autocrine manner by enhancing endothelial cell survival, endothelial cell proliferation, and capillary tube formation; and (iii) causes an intracellular Ca\textsuperscript{2+} rise from intracellular pools which leads to NFκB-directed pro-inflammatory activation and endothelial cell proliferation. These mediators of angiogenesis which are induced by the bacterium probably play a pivotal role in \textit{B. henselae}-induced angiogenesis. When the additional factors from peripheral cells are considered, a model of \textit{B. henselae}-induced angiogenesis emerges (Fig. 19).

Macrophages and monocytes infiltrate lesions caused by BA (49, 50, 64). Macrophages secrete VEGF upon \textit{B. henselae} infection, which probably contributes to angiogenesis during infection (41, 71). We investigated the mechanism by which the macrophage is brought into the site of infection by examining the expression and production of the chemokine MCP-1 from \textit{B. henselae}-infected HMEC-1. MCP-1 is a member of the C-C chemokine family and is produced and secreted by monocytes, fibroblasts, and vascular endothelial cells. MCP-1 then interacts with its CCR2B receptor on monocytes and macrophages to cause chemotaxis (16). MCP-1 can also directly promote angiogenesis. When tumor cells are transfected with \textit{mcp-1} gene and injected into a murine model, angiogenesis is stimulated (68). In addition, MCP-1 implants induce angiogenesis in a rabbit cornea (88). During \textit{B. henselae} infection, MCP-1 released from endothelial cells, most likely in addition to other factors, causes chemotaxis of monocytes and macrophages to the site of infection, thereby promoting an angiogenic state by recruiting the effector cell.
Figure 19. Endothelial cell mediators of angiogenesis during *B. henselae* infection. This model depicts the mediators of angiogenesis that are induced by *B. henselae* from endothelial cells (EC). When ECs are infected with *B. henselae* (BH), MCP-1 is produced and recruits macrophages (Mφ), which secrete VEGF when they are infected. CXCL8 is also produced from ECs, leading to enhanced EC survival and capillary tube formation. BH secretes proteins (BHSP), which induce a Ca\(^{2+}\) spike from intracellular stores and contribute to NFκB-dependent CXCL8 production and EC proliferation. These mechanisms culminate in *B. henselae*-induced angiogenesis.
Bacterial pathogens such as *E. coli*, *Orientia tsutsugamushi*, and *Porphyromonas gingivalis* increase chemokine production and secretion (18, 45, 99). MCP-1 is induced in HMEC-1 in response to *B. henselae* infection (Fig. 4). Both mRNA and protein levels are upregulated; mRNA levels are higher than uninfected controls in *B. henselae*-infected HMEC-1 at 6 and 24 hours after infection, while protein levels in infected cells are higher at 6, 24, and 48 hours after infection. Furthermore, supernatants from *B. henselae*-infected HMEC-1 caused chemotaxis of THP-1 monocytes (Fig. 7). Thus the levels of MCP-1 produced by HMEC-1 in response to *B. henselae* infection *in vitro* are sufficient to function as a chemoattractant for monocytes. Results also reveal that the bacterial factor which causes MCP-1 production is probably a heat stable molecule.

The LPS of *B. henselae* has recently been characterized as containing a lipid A possessing features known to reduce endotoxicity, including a pentaacyl lipid A and a long-chain fatty acid (97). *B. henselae* LPS induces TLR4 1000-fold lower than *Salmonella enterica* sv. Friedenau LPS (97). In addition, LPS from *B. quintana*, which is likely quite similar to *B. henselae* LPS, induces GRO-CINC-1 in rats but not TNF in rats or human whole blood (62). *B. henselae* LPS also does not induce TNF in cats. In this study, the addition of polymyxin B sulfate to *B. henselae* before infection of HMEC-1 did not reduce MCP-1 production; however, polymyxin B sulfate had a significant lowering effect on *E. coli*-induced MCP-1 production (Fig. 4). These data corroborate with the low endotoxicity of LPS from *Bartonella* spp. to imply a limited or nonexistent role for LPS in *B. henselae*-induced MCP-1 production.

Toll-like receptors activated by various microbial products can cause expression and production of chemokines (23, 24, 57), including MCP-1 (76). LPS, a TLR4 agonist,
causes MCP-1 production in a TLR4-dependent manner (89). Most studies confirm that MCP-1 production is TLR4-mediated, and usually caused by LPS in a bacterial infection. However, recently it was discovered that TLR4-deficient and TLR4-competent mice have the same MCP-1 response to infection by *Leishmania major* (6), which is known to cause chemokine production early in infection (40). Our findings indicate that MCP-1 production in response to *B. henselae* infection is not TLR4-dependent (Fig. 5A). In contrast, *E. coli* LPS-induced MCP-1 production was lowered in the presence of a TLR4 monoclonal antibody. These data suggest the possibility of an alternate pathway to TLR4 activation for the MCP-1 production from *B. henselae*-infected HMEC-1. Furthermore, these results again exclude *B. henselae* LPS from a role in MCP-1 production. HMEC-1 express TLR1, TLR3, TLR4, and TLR5 but express TLR2 very weakly, which is why they are unresponsive to TLR2 ligands (25). Thus the MCP-1 production investigated in this study is probably not TLR4- or TLR2-mediated. Other TLR or similar receptor pathways must be investigated to pinpoint the exact mechanism of MCP-1 induction in HMEC-1 in response to *B. henselae*.

The *mcp-1* gene contains binding sites for both NFκB and AP-1 (73, 91), and both transcription factors have been implicated in *mcp-1* expression (18, 94). It has been established that *B. henselae* induces NFκB-dependent upregulation of adhesion molecules in HUVEC independent of LPS (32). The findings from our study suggest the independence of MCP-1 expression and protein production from *B. henselae* LPS. In addition, we used two NFκB inhibitors to determine whether MCP-1 protein production requires NFκB activation. Diverse NFκB inhibitors have been used with HMEC-1 previously in similar experiments (18). PDTC is an antioxidant that inhibits the
phosphorylation of IκB (66, 78) and TPCK inhibits proteosome-dependent degradation of inhibitory peptides (58). Consequently, through the use of these inhibitors, we demonstrated that MCP-1 production caused by B. henselae in HMEC-1 is NFκB-dependent (Fig. 5B).

OMPs of B. henselae are important for pathogenesis (14, 32). Data presented here reveal the ability of B. henselae Houston-1 OMPs, specifically OMPs of low molecular weight, to enhance production of the C-C chemokine MCP-1 from HMEC-1 (Fig. 6A). This upregulation is again independent of LPS, as shown by incubation of OMP-1 with polymyxin B sulfate before addition to HMEC-1 (Fig. 6B). These data point to a heat-stable low molecular weight OMP of B. henselae Houston-1 that contributes at least in part to B. henselae-induced MCP-1 production from endothelial cells. Further studies are needed in order to specify the putative OMP that causes MCP-1 upregulation in endothelial cells.

We have described upregulation of gene expression and protein production of the chemokine MCP-1 in response to B. henselae infection. This stimulation of HMEC-1 is independent of B. henselae LPS and toll-like receptor 4 but dependent on NFκB activity. MCP-1 produced by infected HMEC-1 most likely contributes to the ability of conditioned media from these cells to induce monocyte chemotaxis. The recruitment of macrophages by MCP-1 produced from infected endothelial cells could have broad implications on mechanisms of angiogenesis during this infection. Specifically, the macrophage effector cell which secretes VEGF and other angiogenic factors is brought to the site of infection. Pathogenic angiogenesis provides actively growing target cells for B. henselae in an enriched vascularized microenvironment, and while the specific role of
MCP-1 induction in this phenomenon is not completely understood, we suggest that recruitment of the monocyte/macrophage effector cell is an important component of the pathway.

Angiogenesis is a complex process involving several key steps. These steps include (i) inhibition of endothelial cell apoptosis, (ii) endothelial cell proliferation, (iii) breakdown of the extracellular matrix by MMPs, and (iv) capillary tube formation. CXCL8 can promote each of these steps. Since *B. henselae* upregulates CXCL8 production from endothelial cells (21, 71), we investigated a putative autocrine role for CXCL8 in *B. henselae*-induced angiogenesis.

There are conflicting reports on whether endothelial cells actively proliferate or whether they simply exhibit enhanced survival in the presence of *B. henselae* (43, 77). Endothelial cell proliferation in BA most likely comes from a combination of inhibition of apoptosis and mitogenic stimulation. In addition, endothelial cell proliferation and angiogenesis probably result from the effects of the bacterium on both the endothelial cells and peripheral cells such as epithelial cells and macrophages (41, 71). While this particular study focuses on the autocrine role of CXCL8, a paracrine role should not be overlooked as many cell types produce CXCL8 after infection with *B. henselae* (Fig. 8). Furthermore, the bacterium causes an upregulation of expression of one of the CXCL8 receptors, CXCR2 (Fig. 9). This may represent a mechanism by which the effects of CXCL8 on the endothelial cell are enhanced because the receptor is present at elevated levels. When the fact that CXCL8 production is upregulated from endothelial and other cells is combined with the information that CXCR2 expression is also enhanced during
endothelial cell infection, a model emerges whereby CXCL8 signaling is extremely elevated in the endothelial cell during *B. henselae* infection.

The balance between Bax and Bcl-2 is important for endothelial cell survival or apoptosis. CXCL8 induces an increase in Bcl-2 expression and a decrease in Bax expression, most likely favoring survival over apoptosis in endothelial cells (52). It has been shown that *B. quintana* can modulate the cell-programmed death of HUVEC-C by increasing Bcl-2 expression (54). In this study, we examined expression of two Bcl-2 family members, Bcl-2 (anti-apoptotic) and Bax (apoptotic) in HUVEC by real time RT-PCR. In the presence of *B. henselae*, Bax is decreased and Bcl-2 is increased (Fig. 11A). These increases and decreases are quite dramatic alone; however, when the ratio of Bcl-2 to Bax is considered, the comparison is even more drastic. This is the first report of *B. henselae* mediating Bax and Bcl-2 expression in endothelial cells. In addition, the data reveal a possible role for CXCL8 in this prevention of apoptosis since the presence of anti-CXCL8 abrogates the higher Bcl2 levels and the lower Bax levels induced by *B. henselae* (Fig. 11B, C).

These data also implicate CXCL8 as a mediator of endothelial cell proliferation and capillary tube formation during infection. Both aspects of angiogenesis were decreased in the presence of a CXCL8 neutralizing antibody (Figs. 10, 12). However, other mechanisms are probably also involved in proliferation, including the activity of growth factors such as VEGF from other cells. It has been shown that while *B. henselae* cause endothelial cells to proliferate, this proliferation is inhibited at higher MOIs as a result of a cytotoxic effect from the *B. henselae* TFSS (77). Our proliferation results agreed with this phenomenon; at MOIs above 50, endothelial cell proliferation was
decreased. However, the other aspects of angiogenesis (capillary tube formation, enhanced endothelial cell survival, and CXCL8 production) increased at an MOI of 100 when compared to an MOI of 50 (data not shown). These results suggest that the cytotoxic effect of the products of the virB does not have an effect on expression of Bcl-2 family members or capillary tube formation. Thus the pro-angiogenic effect of B. henselae may consist of a complicated fusion of many host cell and bacterial factors. Nevertheless, CXCL8 seems to play an autocrine and possible paracrine role in B. henselae-induced angiogenesis, representing a mechanism by which the bacterium causes upregulation of CXCL8 thereby increasing its survival by expanding its host cell reservoir. An assessment of the contribution of each of these in vitro components toward the overall angiogenesis mediated by B. henselae is still unfinished, and it will require extensive in vivo and in vitro studies.

B. henselae secreted proteins (BHSP), or conditioned media, have been shown to induce endothelial cell proliferation (59). These proteins are isolated from B. henselae grown on chocolate agar and resuspended in liquid medium for 24 hours. There is a cytotoxic effect from the TFSS of B. henselae, which mediates secretion of BepD into endothelial cells (80), at MOIs above 50. When a virB mutant is used to infect HUVEC, the proliferation is 4-fold higher than with wildtype B. henselae (77). Thus it was determined that the TFSS triggers a cytotoxic effect in HUVEC. The virB promoter is only active inside the cell; B. henselae containing a GFP reporter construct driven by the virB promoter are not green outside of the cell (79). BHSP therefore contain only low levels, if any, of TFSS-transported proteins.
NFκB activation links the upregulation of MCP-1 and CXCL8 during *B. henselae* infection. Thus we investigated upstream of NFκB activation by examining the intracellular Ca\(^{2+}\) response to bacterial secreted proteins. In this study we determined that BHSP in fact cause endothelial cell proliferation (Fig. 14) and that this proliferation is dependent on Ca\(^{2+}\) signaling, since in the presence of the Ca\(^{2+}\) chelator BAPTA/AM HUVEC proliferation was lowered (Fig. 17). Additionally, we demonstrated that BHSP induce a Ca\(^{2+}\) elevation in HUVEC, while a medium control did not have the same effect (Fig. 15). Furthermore, we showed that the origin of the Ca\(^{2+}\) response to BHSP is an intracellular store, since the intracellular Ca\(^{2+}\) store inhibitor thapsigargin abolished the BHSP-induced Ca\(^{2+}\) rise in HUVEC (Fig. 16).

CXCL8 is an important mediator of angiogenesis and is important for HUVEC survival and capillary tube formation during *B. henselae* infection of HUVEC. Since the BHSP induced HUVEC proliferation, we sought to ascertain whether BHSP induce CXCL8 production from HUVEC. In the presence of BHSP, CXCL8 production was raised about four times higher than a medium control (Fig. 18A, P<0.001). However, the CXCL8 levels in the presence of BHSP did not increase above 100 pg/ml (Fig. 18A, B). These CXCL8 levels are lower than those elicited by live *B. henselae* (Fig. 8). Thus the question arises: Are these CXCL8 levels sufficient to cause HUVEC proliferation or is there another proliferative pathway activated by BHSP? In fact, during proliferation assays, HUVEC are seeded at a low density (10\(^3\) HUVEC/well of a 96-well plate) in order to allow for proliferation over 3-4 days. Thus while MOIs of live bacteria take into account the cell numbers, the concentrations of BHSP are determined as µg/ml. Therefore during proliferation assays, higher CXCL8 levels may be elicited as a result of
lower numbers of cells. Unfortunately, since the HUVEC proliferate over 3 or 4 days, measurement of CXCL8 levels would be skewed as there are more cells in BHSP-treated wells. Additionally, the BHSP are present for 3 days and may cause more CXCL8 production over that time course. Consequently, we propose that while the CXCL8 responses to BHSP were not as robust as the response to live B. henselae, these levels may be sufficient to cause HUVEC proliferation. CXCL8 production enhanced by BHSP was lowered in the presence of a Ca\textsuperscript{2+} chelator BAPTA/AM (Fig. 18B). NFκB activation can be mediated by intracellular Ca\textsuperscript{2+} signaling, and BHSP induction of CXCL8 appears to be Ca\textsuperscript{2+}-dependent.

The factor which induces the Ca\textsuperscript{2+} rise and subsequent effects is still unknown. We determined that BHSP contain BadA and GROEL (Fig.13), both which are important during B. henselae infection of endothelial cells (63, 72). BadA binds to the extracellular matrix proteins collagen, laminin, and fibronectin (72). This could be responsible for the Ca\textsuperscript{2+} rise in HUVEC. GROEL is mitogenic for endothelial cells, which may contribute to HUVEC proliferation and CXCL8 production mediated by BHSP. The TFSS mediates CXCL8 production in HUVEC as well (77); perhaps low levels of some of the effectors translocated by the TFSS are present in the BHSP or the components on the bacterial membrane are present in BHSP. Further studies are necessary to determine the factor responsible for proliferation and CXCL8 production, including proteomic analysis of the BHSP and functional assays of these species present in BHSP.

We propose that the BHSP experiments may evolve into an animal model of B. henselae-induced angiogenesis. A rhesus macaque model of B. quintana infection was developed in which the levels of bacteria mimicked human infection (98). No infection
model in mice has been successfully developed; Arvand et.al. showed bacterial presence up to one week after infection in C57/BL6 mice (8), after which the bacteria were cleared. Perhaps the BHSP could be used in an angiogenic model such as an \textit{in vivo} matrigel in mice or the chicken embryo assay, which may circumvent the problems associated with clearance of \textit{B. henselae} during mice infection.

Endothelial cell mediators of angiogenesis induced by \textit{B. henselae} contribute to the overall pathology in \textit{B. henselae} infection. In this study we identified three mediators of angiogenesis induced from the endothelial cell as a result of bacterial factors: MCP-1, which brings the macrophage effector cell into the site of infection; CXCL8, which directly promotes angiogenesis in an autocrine manner; and intracellular Ca$^{2+}$ activity, which contributes to endothelial cell proliferation and NF$\kappa$B activation. These factors and others from peripheral cells culminate in the unique angiogenic lesions seen during \textit{B. henselae} infection in the immunocompromised. A better understanding of how \textit{B. henselae} causes angio-proliferation could lead to the development of improved therapeutics and contribute to the understanding of interactions between intracellular bacteria and host cells.


Presentation of Studies

Publications resulting from these studies include one first-authored paper published, one first-authored paper in press, and one manuscript in preparation. These results were also presented in part as posters at the American Society for Microbiology general meetings and as an oral presentation at the American Society for Microbiology Southeastern Branch meeting. These presentations and papers are listed below.

McCord AM, Cuevas J, Anderson B. *B. henselae* secreted proteins activate intracellular calcium stores in endothelial cells (manuscript in preparation).


*Bartonella henselae* upregulates pro-angiogenic effectors in endothelial cells leading to autocrine promotion of angiogenesis. American Society for Microbiology General Meeting, Orlando, FL.


About the Author

Amy Marie McCord completed her undergraduate education at the University of Florida where she was recognized as a National Merit Scholar and a Florida Bright Futures Scholar and received her B.S. in Microbiology and Cell Science. She entered the University of South Florida in 2001 as a graduate student in the department of Medical Microbiology and Immunology. While at USF, she has received two Outstanding Presentation Awards during the Health Sciences Research Day and was recognized as an alternate for a national travel award from the American Society for Microbiology. She also was awarded Distinction for this dissertation.