Activation of p38 mitogen-activated protein kinase module facilitates *in vitro* host cell invasion by *Rickettsia rickettsii*

Interactions with target membrane molecules and the resultant internalization into host cells are, in general, the first and foremost steps for subsequent host responses and pathogenesis of infectious micro-organisms. For pathogenic bacteria belonging to both spotted fever and typhus subgroups of *Rickettsia* species, which are known for dependence on the nutrient-rich environment of the host cytoplasm and fastidious growth requirements, target cell invasion is absolutely critical for subsequent intracellular replication and intercellular spread. *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, primarily affects the endothelial cell lining of small- and medium-sized vessels leading to disseminated intracellular infection of the vasculature and, consequently, characteristic pathological features during human infections correspond to vascular inflammation, damage and dysfunction (Walker, 2007).

Initial studies using *Rickettsia prowazekii*, the aetiological agent of epidemic typhus, revealed that inactivation of bacteria or alterations in the host cell cytoskeleton adversely affect rickettsial uptake into cultured human umbilical vein-derived endothelial cells, suggesting that the viability of both the host cell and adherent rickettsiae is an essential prerequisite for successful invasion via 'induced phagocytosis' (Walker, 1984). Subsequent investigations by electron microscopy further indicated that rickettsial entry into Vero cells is almost instantaneous (occurring within 3 min of bacterium–host cell contact) and rickettsiae are able to quickly escape from the phagosome into the cytoplasm, presumably prior to phagolysosomal fusion (Teyssie *et al.*, 1995), and likely via a phospholipase activity (Whitworth *et al.*, 2005). An important role for host actin polymerization in rickettsial internalization of non-phagocytic cells has also been suggested. Analysis of proteins known to govern actin dynamics has

identified recruitment of the Arp2/3 complex to the sites of entry foci during *Rickettsia conorii* invasion of Vero cells, and potential coordinated involvement of GTPase Cdc42, phosphoinositide 3-kinase, c-Src and other protein tyrosine kinase activities in the activation of the Arp2/3 complex (Martinez & Cossart, 2004).

Conserved across all eukaryotes, the p38 group of mitogen-activated protein (MAP) kinases is implicated in many physiological processes, including cell growth, differentiation, apoptosis and immune response. A unique feature for activation of the p38 superfamily of enzymes is the requirement for dual phosphorylation of the TXY motif in the activation loop of the catalytic domain, where phosphorylation of tyrosine (Y) usually precedes that of threonine (T) in a two-step reaction. Our laboratory has demonstrated that *in vitro* infection of cultured human endothelial cells with spotted fever rickettsiae (*R. rickettsii* and *R. conorii*) triggers the activation of the p38 signalling module, the specific inhibition of which results in downregulation of infection-induced expression of chemokines [interleukin-8 (CXCL8) and monocyte chemoattractant protein-1 (CCL-2)], and downregulation of the inducible isoform of regulatory bifunctional enzyme cyclooxygenase-2 (Rydkina *et al.*, 2005, 2006). In this report, we have further investigated the possibility of an interrelationship between p38 MAP kinase activation and *R. rickettsii* invasion of host endothelial cells.

Primary cultures of endothelial cells isolated from the umbilical vein of freshly collected human umbilical cords were established and maintained according to previously described procedures (Sahni *et al.*, 1998). Vero C1008 cells were obtained from American Type Culture Collection and maintained in culture as recommended by the supplier. The Sheila Smith strain of *R. rickettsii* was grown in Vero cells and purified on sucrose-

Renografin density gradients (Sahni *et al.*, 1998; Rydkina *et al.*, 2005). The pyridinyl imidazole SB 203580 was used as a selective inhibitor of p38 MAP kinase. This compound does not inhibit other MAP kinase homologues c-Jun-N-Kinase (JNK) and p42 MAP kinase, but has previously been shown to specifically inhibit *R. rickettsii*-induced activation of p38 (Rydkina *et al.*, 2005). SB 202474, a structurally similar compound with no inhibitory activity towards p38 activity, was used as a negative control in all experiments. To determine the extent of rickettsial invasion into host cells, we employed three independent approaches: immunofluorescent staining of rickettsiae followed by quantification of the percentage of cells infected and the number of intracellular bacteria (Sahni *et al.*, 1998), plaque formation assay (Rydkina *et al.*, 2005) and citrate synthase gene (*gltA*)-based real-time PCR (Rydkina *et al.*, 2007). Plaque formation assays were carried out using Vero cell monolayers, whereas indirect immunofluorescence and quantitative PCR-based enumeration was conducted using human endothelial cells. The durations for inhibitor treatment as well as *R. rickettsii* infection for both cell types were identical; each experimental condition was repeated to achieve a minimum of three replicates, and results obtained were subjected to statistical evaluation as described previously (Rydkina *et al.*, 2005, 2007).

First, we compared the level of infection in endothelial cells grown to confluence on sterile coverslips and incubated for 3 h with *R. rickettsii* in the presence and absence of 10 μ M SB 203580 (SB 80) and 10 μ M SB 202474 (SB 74) in the culture medium. Indirect immunofluorescent staining using an anti-*R. rickettsii* antiserum and a rhodamine-conjugated compatible secondary antibody revealed that in the presence of specific p38 inhibition via SB 80 the number of adherent/intracellular rickettsiae was

comparatively lower than in those endothelial cells infected in culture medium alone or in the presence of SB 74, an inactive structural analogue of SB 80 (Fig. 1). The extent of plaque formation on confluent monolayers of Vero cells was next determined under similar experimental conditions. After 3 h exposure to viable *R. rickettsii*, which was followed by thorough washing and layering of the cell monolayer with 0.5 % agarose in culture medium, visibly distinct plaques resulted due to infection-induced lysis of host cells on day 7 post-infection. Again, cell treatment with SB 74 during the process of initial contact and invasion had no effect on the ability to induce plaque formation. Presence of SB 80 during infection, however, led to a significant decrease (36 ± 7 %) in the number of rickettsial plaques ($P=0.017$, when compared to corresponding untreated controls and SB 74-treated cells) (Fig. 2). Next, the number of intracellular rickettsiae in endothelial cells infected in the presence of p38 inhibition was quantified by a real-time PCR-based approach using a rickettsial *gltA*-specific primer pair and a TaqMan probe to determine the mean copy number of *gltA*

in relation to that of the housekeeping gene GAPDH. Again, treatment of endothelial cells with SB 80 significantly reduced the invasion of *R. rickettsii* ($\sim 38 \pm 12$ % inhibition in comparison to cells infected without any treatment; $P=0.025$). Infection in the presence of SB 74, a negative control for SB 80, had no significant effect on rickettsial invasion when compared to controls ($\sim 17 \pm 13$ % inhibition; $P=0.21$) (Fig. 2). Thus, when directly compared to infection in the presence of SB 74, SB 80 caused an approximately 25 % decrease in the invasion of *R. rickettsii*. Taken together, these results suggest that p38 MAP kinase activation in response to infection plays an important role in facilitating host cell invasion by *R. rickettsii*.

As pathogenic bacteria with an obligate intracellular lifestyle, internalization by host cells, followed by replication within the cytoplasm, has important implications in the establishment, progression and dissemination of infection for various *Rickettsia* species. It is well known that among major immunodominant surface antigens, rickettsial outer membrane protein A (rOmpA) and rOmpB are expressed by spotted fever group

rickettsiae. Inhibition studies using mAbs against both of these proteins are clearly suggestive of a critical role for rOmpA in *R. rickettsii* adhesion to host L929 cells (Li & Walker, 1998). More recent proteomic characterization of rickettsial adhesins by two-dimensional electrophoresis coupled with MS analysis has revealed two highly conserved, putative rickettsial ligands, one of which was identified as a C-terminal β -peptide of rOmpB, whereas the other was a protein of unknown function encoded by ORFs RC1281 in *R. conorii* and RP828 in *R. prowazekii* (Renesto *et al.*, 2006). Interestingly, interactions between rOmpB and Ku70, a DNA binding component of DNA-dependent protein kinase, have recently been implicated in *R. conorii* entry into non-phagocytic host cells (Martinez *et al.*, 2005). The potential contribution of host cell signalling mechanisms in facilitating internalization of rickettsiae, however, remains relatively poorly defined. *R. conorii* uptake into mammalian cells has been shown to be associated with tyrosine-specific phosphorylation of a number of host proteins, including focal adhesion kinase, and efficient invasion is dependent not only on actin polymerization, but also on activities of phosphoinositide 3-kinase

Fig. 1. Immunofluorescent staining for *R. rickettsii* (Rr) in endothelial cells infected in the presence and absence of specific p38 kinase inhibitor SB 203580 (SB 80) or its inactive structural analogue SB 202474 (SB 74). Cells grown to ≥ 80 % confluence on sterile plastic coverslips were treated with SB 80 or SB 74 at a final concentration of 10 μ M for 30 min prior to and during 3 h infection with *R. rickettsii*. After fixation, coverslips, including those with uninfected endothelial cells (control), were incubated with an anti-*R. rickettsii* primary antibody followed by a rhodamine-conjugated secondary antibody. The level of intracellular infection was then determined by counting a minimum of 300 infected cells with or without SB 74/SB 80 treatment. Representative photomicrographs for each experimental condition are shown. Figures in parentheses underneath the images represent the number of intracellular rickettsiae per infected cell, calculated as the mean $\pm$ SE from three separate experiments. As determined by Student's *t*-test, *P* values for cells treated with SB 74 or SB 80 in relation to infection alone are also shown.

Fig. 2. Effect of selective p38 kinase inhibition on internalization of *R. rickettsii* into host cells. Vero C1008 and endothelial cells were either left untreated or incubated for 30 min with SB 74 and SB 80, after which infection was carried out for 3 h in the continued presence of inhibitor compounds. The level of rickettsial invasion was then determined by plaque formation assay in Vero cells and by real-time quantitative PCR (qPCR) in endothelial cells. The number of internalized rickettsiae in cells receiving no treatment (white bars) was assigned an absolute value of 100 and compared with cells infected with *R. rickettsii* in the presence of SB 74 (grey bars) or SB 80 (black bars). The data are presented as the mean $\pm$ SEM from at least three independent experiments. Asterisks indicate a statistically significant decrease in comparison to infection alone ($P \leq 0.05$).

and protein tyrosine kinase (Martinez & Cossart, 2004).

Three major MAP kinase cascades, namely extracellular signal-regulated kinases (ERKs), c-Jun-N-terminal kinases and p38 MAP kinases, have been identified and characterized in detail. These subfamilies can be activated simultaneously or independently in a variety of situations to constitute a critical component of a 'central' regulatory mechanism. Involvement of MAP kinases in host cell responses to infection with a number of pathogenic bacteria has been established (Rydkina *et al.*, 2005). Available evidence further implicates MAP kinases in the regulation of cytoskeletal organization in various systems, including invasion of host epithelial cells by *Campylobacter jejuni* and other invasive enteric bacterial pathogens (Hu *et al.*, 2006). Similarly, uptake of *Chlamydia pneumoniae* by HEp-2 cells is effectively blocked by U0126, a specific inhibitor of MEK1/2-dependent activation of ERK1/2 (Coombes & Mahony, 2002). Further, interaction of *Neisseria meningitidis* with human brain microvascular endothelial cells results in phosphorylation and activation of JNK1 and JNK2 and p38 MAP kinase, as well as their direct substrates c-Jun and MAP kinase-activated kinase-2, respectively. In this case, inhibition of JNK via SP600125 reduces the invasion of meningococci, whereas p38 inhibitors

SB202190 and SB203580 have no effect (Sokolova *et al.*, 2004).

Using *in vitro* infection of cultured human endothelial cells, the preferred target cell type in patients with confirmed diagnosis of spotted fever rickettsioses and in established *in vivo* models of infection, we have shown selective activation of p38 MAP kinase during infection with *R. rickettsii* and *R. conorii* (Rydkina *et al.*, 2005, 2007). The present communication provides what is believed to be the first evidence that *R. rickettsii* internalization into endothelial cells likely involves signalling through the p38 module of MAP kinases. This observation corroborates our earlier findings that adherence of viable rickettsiae to the host cell surface is apparently sufficient to initiate intracellular signalling events to induce p38 phosphorylation and activation (evident as early as 15 min after contact between endothelial cells and *R. rickettsii*). Such an immediate-early response, in turn, facilitates the process of bacterial internalization into host cells. It is important to emphasize, however, that partial blockade of invasion in the presence of p38-specific inhibition suggests the participation of multiple signalling mechanisms, and supports the possibility of rickettsial interactions with other cell surface receptors in addition to Ku70 (Martinez *et al.*, 2005). Acquiring detailed knowledge of signalling pathways exploited by pathogenic *Rickettsia* species to

invade different mammalian cells should not only allow better understanding of host tissue tropism, but should also lead to the development of novel strategies aimed at preventing invasion as the fundamental pathogenesis mechanism.

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