Involvement of endothelial ephrin-B2 in adhesion and transmigration of EphB-receptor-expressing monocytes

Dennis Pfaff 1, Mélanie Héroult 1, Maria Riedel 1, Yvonne Reiss 2, Robert Kirmse 3, Thomas Ludwig 3, Thomas Korff 4, Markus Hecker 4 and Hellmut G. Augustin 1,*

1 Joint Research Division Vascular Biology, Medical Faculty Mannheim, University of Heidelberg, and German Cancer Research Center (DKFZ-ZMBH Alliance), Heidelberg, Germany
2 Institute of Physiology and Pathophysiology, Division of Cardiovascular Physiology, University of Heidelberg, Germany
3 Group Microenvironment of Tumor Cell Invasion, German Cancer Research Center (DKFZ), Heidelberg, Germany
4 Institute of Physiology and Pathophysiology, Division of Cardiovascular Physiology, University of Heidelberg, Germany

*Author for correspondence (e-mail: augustin@angiogenese.de)

Accepted 6 August 2008
Journal of Cell Science 121, 3842-3850 Published by The Company of Biologists 2008
doi:10.1242/jcs.030627

Summary
The vascular endothelium is a crucial interface that controls the recruitment of circulating leukocytes. Based on the luminal expression of the ephrin-B2 ligand by endothelial cells (ECs) and the expression of EphB receptors (EphBRs) by circulating monocytes, we hypothesized that EphBR-ephrinB interactions are involved in monocyte adhesion. Adhesion experiments with monocytes and ECs performed on ECs that overexpressed either full-length ephrin-B2 or cytoplastically truncated ephrin-B2 (ΔC-ephrin-B2). Atomic force microscopy confirmed similar adhesive strengths of EphBR-expressing J774 cells to ECs that either overexpressed full-length ephrin-B2 or truncated ΔC-ephrin-B2 (1-minute interaction). Yet, adhesion experiments under static or flow conditions for 30 minutes demonstrated the preferential adhesion of monocytes to ECs that overexpressed full-length ephrin-B2 but not to ΔC-ephrin-B2 or to ECs that had been mock transduced. Adhesion was blocked by ephrin-B2-specific and EphBR-specific antibodies. Correspondingly, adhesion of EphB4-receptor-overexpressing monocytes to ephrin-B2-positive ECs was further augmented. Trafficking experiments of cell-surface molecules revealed that, prior to internalization, the resulting EphB4-receptor–ephrin-B2 complex translocated from the luminal surface to inter-endothelial junctions. Lastly, full-length ephrin-B2 in ECs was also involved in monocyte transmigration. Collectively, our study identifies a role of EphBR-ephrinB interactions as a new step in the cascade of events leading to monocyte adhesion and transmigration through the vascular endothelium.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/22/3841/DC1

Key words: Endothelial cells, EphB4 receptor, Ephrin-B2 ligand, Adhesion, Transmigration

Introduction
The vascular endothelium forms an interface between the circulation and the different body compartments. As such, it controls the trafficking of leukocytes. The recruitment of circulating cells is orchestrated by the coordinated activities of adhesive and chemotactic signals. The sequential activities of selectins, integrins and IgG-superfamily molecules in concert with chemokines control leukocyte tethering, rolling, firm adhesion and subsequent transmigration through the vascular endothelium (Vestweber, 2007). The specific repertoire of adhesion molecules and chemotactic signals establishes the site and cell-type-selective vascular ‘area code’ necessary for spatiotemporal control of leukocyte trafficking (Vestweber, 2007). Whereas the major players of leukocyte trafficking have been well established, additional adhesive signals may be involved in the fine tuning of the vascular address code. Most notably, the steps leading from firm leukocyte adhesion to transmigration are still poorly understood.

Ephrin (Eph) receptors and their corresponding ephrin ligands are cell surface molecules that elicit positional guidance cues on corresponding receptor and ligand expressing cells that get into juxtapositional contact (Arvanitis and Davy, 2008; Kullander and Klein, 2002; Wilkinson, 2001). Originally identified as neuronal pathfinding molecules, Eph receptors and ephrin ligands are now recognized as being widely expressed controlling cell-cell interactions of multiple cell types (Kullander and Klein, 2002; Pfaff et al., 2006). Vascular functions of Eph receptors and their ligands were identified a decade ago when genetic loss-of-function experiments revealed surprising roles of the Eph-receptor ligand ephrin-B2 (also known as EFNB2) and its receptors EphB2, EphB3 and EphB4 in controlling arteriovenous assembly and differentiation (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Ephrin-B2 is selectively expressed by arterial and angiogenically activated endothelial cells (ECs). By contrast, the EphB4 receptor is preferentially expressed by venous ECs. As such, ephrin-B2 and the EphB4 receptor were established as the first markers to discriminate between arteries and veins, which has stimulated research into further defining the molecular repertoire of arterial vs venous ECs (Augustin and Reiss, 2003; Torres-Vazquez et al., 2003). EphB receptors (EphBRs) are classical receptor tyrosine kinases (RTKs) and interact with their corresponding ephrinB ligands that themselves act as transmembrane tyrosine kinase signaling molecules. EphBR-ephrinB interactions are capable of mediating bi-directional signaling events upon cell-cell contact, either into the
receptor-expressing cell as ‘forward signaling’ or into the ligandexpressing cell as ‘reverse signaling’.

Vascular morphogenic functions of EphBRs and their corresponding ephrin-B2 ligand in development are well established. Yet, much less is known about their roles in the adult vasculature. The arteriovenous asymmetrical expression patterns of ephrin-B2 and EphB4 are maintained in the adult (Diehl et al., 2005; Korff et al., 2006). Likewise, ephrin-B2 expression is prominently upregulated during adult neovascularization (Gale et al., 2001; Hayashi et al., 2005; Shin et al., 2001) and arteriogenic vessel remodeling (Korff et al., 2008). EphBR–ephrin-B2 interactions have recently been implicated in regulating inflammation (Ivanov et al., 2005; Yuan et al., 2004). Expression of EphBRs and their ligand ephrin-B2 has also been described in different circulating cell populations, including platelets (Prevost et al., 2002), T-cells (Yu et al., 2003), dendritic cells (de Saint-Vis et al., 2003), bone marrow hematopoietic cells (Okubo et al., 2006) and endothelial progenitor cells (Foubert et al., 2007).

We have shown that activation of endothelial ephrin-B2 reverse signaling exerts pro-adhesive and pro-migratory effects on ECs, whereas forward EphB4 signaling interferes negatively with the angiogenic cascade (Füller et al., 2003). Expression-profiling experiments in vitro and in vivo have established the microenvironmental control of EC-expressed ephrin-B2 by cytokines, contact with smooth muscle cells and biomechanical forces (Goetttsch et al., 2004; Hayashi et al., 2005; Korff et al., 2006; Korff et al., 2008). Importantly, ephrin-B2 was found to be apically expressed in adult ECs (Korff et al., 2006). The apical expression of ephrin-B2 in ECs, in combination with the detection of EphB-positive cells in the circulation prompted us to hypothesize that EphBR–ephrin-B2 interactions have a role in controlling leukocyte recruitment to the endothelium. The rationale for these experiments was also stimulated by our recent observation that arteriogenic vessel remodeling is associated with the induction of ephrin-B2 expression and concomitant monocyte recruitment (Korff et al., 2008). The present study was consequently aimed at systematically exploring this hypothesis at the cellular level. The experiments have solidly established a role of the EphBR–ephrin-B2 system in controlling monocyctic cell adhesion to ECs and their subsequent transmigration.

**Results**

**Expression of EphBRs in hematopoietic cells and monocytic cell lines**

Guided by our previous finding that ephrin-B2 is expressed on the luminal side of ECs and is upregulated during angiogenesis (Korff et al., 2006), as well as the arteriogenesis-associated upregulation of ephrin-B2 with enhanced monocyte transmigration (Korff et al., 2008), we set out to systematically analyze the cellular mechanisms of ephrin-B2- and EphBR-mediated adhesive interactions between ECs and leukocytes. Fluorescence-activated cell sorting (FACS) analysis of leukocyte EphB4 expression identified several EphBR-positive leukocyte populations (Fig. 1). Mouse bone-marrow-derived hematopoietic cells, mouse peripheral blood leukocytes, mouse splenocytes and mouse macrophages (F4/80+) were all found to express EphBRs (Fig. 1A). EphBRs were also expressed by human CD14+ cells, the human leukemic monocyte cell line U937 and the murine macrophage cell line J774 (Fig. 1B). The detailed analysis of EphBR expression by FACS experiments identified the high-affinity ephrin-B2 receptors EphB2 and EphB4 (Chrencik et al., 2006) to be expressed on the cell surface of CD14+ cells (Fig. 1C).

**Ephrin-B2 expression in ECs mediates the adhesion of monocytes**

Previous research has established attractive functions of EC-expressed ephrin-B2 (Füller et al., 2003; Hamada et al., 2003), supporting the hypothesis that ephrin-B2-positive ECs exert pro-adhesive effects on EphB-positive circulating mononuclear cells. We consequently analyzed the adhesive properties of ECs that were retrovirally transduced to express different forms of ephrin-B2, i.e. full-length, cytoplasmically truncated (ΔC-ephrin-B2) ephrin-B2. All transduced cells also expressed GFP via an IRES site (see Materials and Methods). Transduced ECs were probed for morphological changes (live imaging by using the GFP signal), and differences in their ephrin-B2 and ΔC-ephrin-B2 expression patterns were assessed by immunoprecipitation using EphB4 linked to the Fc portion of human IgG (EphB4-Fc) followed by western blot analysis using an anti-ephrin-B2 antibody (Fig. 2A,B). Ephrin-B2 and EphB4 expressions in transduced human umbilical vein endothelial cells (HUVECs) (mock-transduced, Ephrin-B2- or ΔC-ephrin-B2-transduced) were traced by reverse transcription (RT)-PCR and western blotting (supplementary material Fig. S1A,B). No changes in EphB4 phosphorylation was detected in the transduced cells (supplementary material Fig. S1B). Both, full-length ephrin-B2 as well as ΔC-ephrin-B2 were expressed on the cell surface and no obvious morphological changes were detectable in the transduced cells expressing the different constructs (supplementary material Fig. S1C). Protein levels in cells expressing transduced ephrin-B2 were compared with those in

---

**Fig. 1.** FACS analysis of EphB4 expression in different hematopoietic cells. (A) FACS analysis of mouse bone-marrow-derived hematopoietic cells, peripheral blood leukocytes, splenocytes and macrophages (F4/80+) assessing EphB4 expression by ephrin-B2-Fc binding (gray area) compared with Fc-control staining (white area). (B) FACS analysis of human monocytes (CD14+) and two monocytic cell lines (human U937, mouse J774) assessing EphB4 expression by staining for ephrin-B2-Fc (gray area) compared with Fc control staining (white area). (C) FACS analysis of human monocytes (CD14+) that express EphB2 and EphB4 by using relevant antibodies (gray area) compared with IgG-control (white area).
freshly isolated lung ECs expressing endogenous ephrin-B2. We found that ephrin-B2-transduced ECs expressed ephrin-B2 at levels that were comparable with those found in ECs in vivo (supplementary material Fig. S2). Likewise, no changes in the proliferative behavior of the transduced cells were observed (Fig. 2C). Corresponding to in vivo ephrin-B2 expression on the luminal side of ECs (Korff et al., 2006), cultured ECs that overexpressed ephrin-B2 presented ephrin-B2 uniformly on their apical cell surface (Fig. 2D). Similarly, ΔC-ephrin-B2 was apically expressed on the cell surface of cultured ECs (data not shown).

U937 and J774 cells preferentially adhered to ephrin-B2-overexpressing HUVECs compared with mock-transduced or ΔC-ephrin-B2-overexpressing ECs (Fig. 3A,B), suggesting a requirement of the cytoplasmic domain of ephrin-B2 for this adhesion process. By contrast, A375 melanoma cells that expressed EphB1 and EphB2 receptors did not show this pro-adhesive phenotype (data not shown). Adhesive interactions of EphBR–ephrin-B2 controlled early steps of the leukocyte adhesion cascade, as evidenced by the observation that long-term (16 hours) adhesion of J774 cells led to adhesion similar to that in mock-transduced, ephrin-B2- and ΔC-ephrin-B2-overexpressing HUVECs (Fig. 3C). Similarly, adhesion experiments under laminar flow revealed the same pro-adhesive phenotype of J774 cells adhering to ephrin-B2-overexpressing HUVECs than mock-transduced or ΔC-ephrin-B2-overexpressing cells (Fig. 3D; supplementary material Movies 1–3).

Single-cell force spectroscopy revealed the adhesive strength between monocytes and transduced HUVECs

Single-cell force spectroscopy (SCFS) was used to study the initial binding properties of mock-transduced, ephrin-B2- and ΔC-ephrin-B2-overexpressing HUVECs to EphBR-expressing monocytes. GFP-positive ECs were selected and used for SCFS. The principle of SCFS is represented in Fig. 4A,B. The maximum unbinding force and unbinding work of EphBR-expressing J774 cells from ephrin-B2- and ΔC-ephrin-B2-overexpressing HUVECs following an adhesion period of 1 minute was significantly higher than the force required to remove rapidly adhering cells from mock transduced cells (Fig. 4C). Yet, the adhesive strength of ephrin-B2 and ΔC-ephrin-B2 was identical (Fig. 4C).

EphBR–ephrin-B2-dependent adhesion between monocytes and ECs can be blocked

Pre-treatment of J774 cells with an anti-EphB2 antibody or an anti-EphB4 antibody led to a significant decrease in adhesion to ephrin-B2-overexpressing HUVECs (Fig. 5A). Pre-treatment of J774 cells with both antibodies further blocked adhesion (Fig. 5A). Accordingly, pre-treatment of HUVEC monolayers with an anti-ephrin-B2 antibody led to a significant decrease in U937 cell adhesion to ephrin-B2-overexpressing HUVECs (Fig. 5B). Baseline adhesion of mock-transduced HUVECs was unaffected (Fig. 5A,B). Taken together, endogenously expressed EphBRs of monocytes were involved in the adhesion to ephrin-B2-overexpressing ECs.

Fig. 2. Expression of ephrin-B2 and ΔC-ephrin-B2 in HUVECs and PAE cells. (A) Bright-field (BF) imaging and GFP live imaging of mock-transduced, ephrin-B2- and ΔC-ephrin-B2-overexpressing HUVECs. Scale bar: 50 μm. (B) Ephrin-B2 and ΔC-ephrin-B2 overexpression in porcine aortic ECs (PAEC) detected by immunoprecipitation with EphB4-Fc followed by western blotting using anti-ephrin-B2 antibody. (C) MTT-proliferation assay of transduced HUVECs assayed over a period of 96 hours. (D) Confocal microscopy analysis of ephrin-B2 expressed in ECs validating its apical expression. Scale bar: 10 μm.

Fig. 3. Preferential adhesion of EphBR-expressing monocytes to ephrin-B2-overexpressing ECs. (A,B) Adhesion of human U937 or mouse J774 cells to HUVEC monolayers. Adhesion was quantified after 30 minutes of incubation on a rocking platform. U937 and J774 cells adhered preferentially to ephrin-B2-overexpressing HUVECs, but not to ΔC-ephrin-B2-overexpressing or mock-transduced HUVECs. (C) Adhesion of mouse J774 cells to HUVEC monolayers for 16 hours leading to saturation-level adhesion showing no difference to the three transduced monolayers. (D) Adhesion of J774 cells to HUVEC monolayers under flow conditions. Adhesion was quantified after 30 minutes of incubation under flow. J774 cells adhered preferentially to ephrin-B2-overexpressing HUVECs, but not to ΔC-ephrin-B2-overexpressing or mock-transduced HUVECs. Values are expressed as the mean ± s.d. of one representative experiment of two independent experiments with similar results (**P < 0.01).
Ephrin-B2 mediates monocyte adhesion

Ephrin-B2 is a molecule that plays a role in the adhesion of monocytes. This process involves the interaction of Ephrin-B2 with EphB receptors on the surface of endothelial cells (ECs).

Next, we hypothesized that the pro-adhesive phenotype of endogenous EphB-expressing monocytes to ephrin-B2 does not just depend on the reverse signaling of ephrin-B2, but also involves mechanisms that depend on the forward signaling of EphB receptors. To address this question, J774 cells overexpressing EphB4 were generated and compared with mock-transduced cells. Protein expression was assessed by western blot analysis (Fig. 6A). EphB4-GFP or ΔC-EphB4-GFP were expressed on the cell surface of J774 cells as confirmed by surface trypsinization followed by western blot analysis (data not shown). The ΔC-EphB4-GFP mutant lacked the cytoplasmic domain of EphB4 and was therefore signaling incompetent. J774 cells that expressed full-length or truncated EphB4, as well as mock-transduced J774 control cells were allowed to adhere to ephrin-B2-overexpressing HUVEC monolayers and to mock-transduced HUVEC (Fig. 6B). Overexpression of EphB4 in J774 cells led to a significant increase (1.6-fold) in adhesion to ephrin-B2-overexpressing HUVECs compared with mock-transduced HUVECs. Adhesion of J774 EphB4 cells was dependent on the cytoplasmic domain of EphB4, as evidenced by similar adhesion of ΔC-EphB4 J774 cells and mock-transduced J774 cells.

EphBR–ephrin-B2 complexes are junctionally translocated prior to internalization

EphBR-positive monocytes adhered preferentially to ECs that overexpressed full-length ephrin-B2, but not to those expressing truncated ΔC-ephrin-B2 (Fig. 3A,B). We therefore compared the fate of ephrin-B2 and ΔC-ephrin-B2 expressed on the EC surface following their binding to the receptor. EC monolayers were stimulated with the soluble EphB4 (EphB4-Fc) and the cellular location of the resulting complex was traced over time. Monolayers were at different time points surface-trypsinized to discriminate between cell-surface-presented and internalized EphB4–ephrin-B2 complexes. Non-stimulated and non-trypsinized cells expressed ephrin-B2 and ΔC-ephrin-B2 on the respective cells (Fig. 7A,B, first lane). Trypsin treatment of unstimulated cells removed ephrin-B2 and ΔC-ephrin-B2 almost completely, indicating that both molecules were primarily expressed on the cell surface (Fig. 7A,B, second lane). Stimulation of ephrin-B2-overexpressing cells with EphB4-Fc induced the internalization of the resulting EphB4–ephrin-B2 complex, as evidenced by the presence of ephrin-B2 in surface-trypsinized cells. Internalized ephrin-B2 was first detected after 15 minutes and maximum internalization could be

Fig. 4. Single-cell force spectroscopy (SCFS) to study the adhesive strength of EphBR–ephrin-B2 interactions in a cellular context. (A) Model of SCFS. An individual J774 cell was attached to a cantilever and moved towards the surface of HUVECs that overexpressed ephrin-B2 or ΔC-ephrin-B2, or towards mock-transduced HUVECs. The cantilever was then moved in the opposite direction to separate the cells following a cell-cell contact time of 1 minute. The adhesive force was calculated from the deflection of the cantilever and its spring constant. (B) J774 cell attached to the tip of the cantilever (left). Cantilever above the HUVEC layer (right). (C) Average maximum unbinding force and work needed to detach the J774 cell from ephrin-B2- or ΔC-ephrin-B2-transduced HUVECs or from mock-transduced HUVECs. Statistical analysis using a one-way ANOVA followed by a multiple pairwise comparison (Holm-Sidak) revealed that there was no significant difference between either ephrin-B2 or ΔC-ephrin-B2 whereas the difference between mock and both experimental groups was significant (*P<0.05). Average values were calculated from n=24-30 individual force-distance measurements. The average work required to separate the cells completely from each other was represented by the area under curve of the force plot. Scale bars: 50 μm.

Fig. 5. Blocking of monocyte adhesion by using specific antibodies against EphBRs and ephrin-B2. (A) Pre-incubation of J774 cells with an anti-EphB2 or anti-EphB4 antibodies prior to seeding on HUVEC monolayers blocked the preferential adhesion to ephrin-B2-overexpressing HUVECs. Blocking with anti-EphB2 and anti-EphB4 antibodies further augmented the effect. (B) Pre-incubation of the HUVEC monolayer with anti-ephrin-B2 antibody prior to U937 cell seeding blocked the preferential adhesion to ephrin-B2-overexpressing HUVECs. Values are expressed as the mean ± s.d. of one out of three independent experiments with similar results (*P<0.05; **P<0.01).
junctional translocation of cell-surface-expressed ephrin-B2 and Stimulation of cells with EphB4-Fc for 20 minutes induced the expressed uniformly on the cell surface of cultured cells (Fig. 7C). All three cell populations expressed endogenous EphB4 as reflected by a faint, but detectable band at 130 kDa (middle arrow). Full-length EphB4-GFP (>130 kDa) and truncated Δ-EphB4-GFP (<130 kDa) are marked accordingly. Actin served as loading control. (B) Adhesion of mock-transduced, and EphB4- and Δ-EphB4-overexpressing J774 cells to ephrin-B2-overexpressing and mock-transduced HUVECs. Cells were incubated for 30 minutes on a rocking platform. Endogenous EphB4 expression facilitated the preferential adhesion to ephrin-B2-overexpressing HUVECs compared with mock-transduced HUVECs, confirming the findings obtained with J774 cells (Fig. 3). Overexpression of full-length EphB4 but not Δ-EphB4 in J774 cells further augmented the preferential adhesion of J774 cells to ephrin-B2-expressing HUVECs. Values are expressed as the mean ± s.d. of one out of three independent experiments with similar results (**P<0.01).

observed between 30 and 60 minutes (Fig. 7A, lanes 3-6). By contrast, Δ-ephrin-B2, albeit being correctly expressed at the cell surface (Fig. 7B, lane 2 compared with lane 1), was not internalized following stimulation with EphB4-Fc (Fig. 7B, lanes 3-6).

Following the biochemical analysis of EphB4–ephrin-B2 complex trafficking, we performed staining experiments to analyze in detail the spatiotemporal pattern of EphB4–ephrin-B2-complex formation and internalization. Cytochemical analysis confirmed the biochemical findings, in that both ephrin-B2 and Δ-ephrin-B2 were expressed uniformly on the cell surface of cultured cells (Fig. 7C). Stimulation of cells with EphB4-Fc for 20 minutes induced the junctional translocation of cell-surface-expressed ephrin-B2 and Δ-ephrin-B2 (Fig. 7C). A 60-minute EphB4-Fc stimulation of cells that overexpressed full-length ephrin-B2 resulted in a granular cytoplasmic staining pattern of ephrin-B2, reflecting the internalization of the EphB4–ephrin-B2 complex (Fig. 7C). By contrast, Δ-ephrin-B2 was still found at inter-endothelial junctions following prolonged stimulation with EphB4-Fc, reflecting the inability to internalize the resulting EphB4–Δ-ephrin-B2 complex. Confocal microscopic analysis of ephrin-B2- and Δ-ephrin-B2-overexpressing ECs revealed an unaltered location of the adherens junction molecule VE-cadherin (Fig. 7D), confirming an unchanged vascular integrity. VE-cadherin did not colocalize with ephrin-B2 and Δ-ephrin-B2 (Fig. 7D). Collectively, our data established that cell-surface-expressed ephrin-B2 translocated into cell junctions upon receptor engagement prior to the cytoplasmic domain-dependent internalization of the resulting complex.

Monocytes transmigrate preferentially through ephrin-B2-expressing EC monolayers

On the basis of the observed translocation of ephrin-B2 to inter-endothelial junctions, we hypothesized that ephrin-B2 expressed in ECs is functionally involved in the transmigration of EphBR-positive cells. Long-term adhesion experiments have shown that maximum adhesion of J774 cells resulted in similar numbers of J774 cells adhering to ECs that overexpressed full-length ephrin-B2 and Δ-ephrin-B2 or had been mock-transduced (Fig. 3C). Next, HUVECs overexpressing ephrin-B2 or Δ-ephrin-B2, or those that had been mock-transduced, were grown to confluence on Transwell filters prior to seeding of J774 cells. Confluence of the HUVEC monolayers was controlled by calcein staining (Fig. 8, top). J774 cells were allowed to transmigrate for 24 hours and were counted on the lower side of the Transwell membrane. The number of J774 cells migrating through ephrin-B2-overexpressing HUVEC monolayers was significantly higher than those migrating through Δ-ephrin-B2-overexpressing cells and mock-transduced HUVEC monolayers (Fig. 8, center). The ephrin-B2 antibody, which blocked short-term adhesion, did not block transmigration (data not shown). Collectively, these results point towards a role of ephrin-B2 – when expressed in ECs – in the transmigration of EphBR-positive cells.

Discussion

Eph receptors form the largest family of receptor tyrosine kinases, consisting of ten A- and six B-class receptors that bind their corresponding A- or B-class ephrin ligands (Pfaff et al., 2006). EphrinA ligands are GPI-anchored molecules, whereas ephrinB ligands are transmembrane molecules that are capable of transducing signals through their cytoplasmic tail. The EphBRs and ephrinB-class ligands have multiple phosphorylation sites in their cytoplasmic domain and a PDZ-binding domain at their cytoplasmic C-terminal end that interacts with molecules important for downstream signaling (Ran and Song, 2005; Su et al., 2004). Eph receptors and ephrin ligands have originally been identified as neuronal pathfinding molecules that transduce attractive and repulsive signals upon contact of cells that express corresponding receptors and ligands (Kullander and Klein, 2002); they thereby act as crucial regulators of neuronal connectivity.

Eph receptors and ephrin ligands have been identified as being widely expressed and as controlling cell-cell interactions of numerous cell types, including cells of the vascular system (Adams, 2002). The research focus of vascular Eph receptors and ephrin ligands has primarily been on developmental issues, unravelling the role of B-class Eph receptors and ephrin ligands in arteriovenous differentiation (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998), interactions between EC and smooth-muscle cells (Foo et al., 2006) and lymphangiogenesis (Makinen et al., 2005). Studies of interactive mechanisms between Eph receptors and ephrin ligands in the adult vascular system primarily concentrated on their role in tumor angiogenesis and tumor progression (Erber et al., 2006; Kertesz et al., 2006; Martiny-Baron et al., 2004; Noren et al., 2004).

We have previously observed that ephrin-B2 expression in ECs is under microenvironmental control and that ECs express ephrin-B2 on their luminal side (Korff et al., 2006). Other studies have established shear stress and hypoxic stress as major determinants of expression of ephrin-B2 in ECs (Goetttsch et al., 2004; le Noble et al., 2004; Suenobu et al., 2002; Vihanto et al., 2005). The luminal
expression of ephrin-B2 has prompted us to hypothesize that ephrin-B2 presented in ECs has a role in the recruitment of leukocytes by interacting with EphBRs expressed by circulating cells. This hypothesis was also supported by our recent observation that arteriogenic vascular remodeling is associated with the induction of ephrin-B2 expression and concomitant monocyte recruitment (Korff et al., 2008). We also investigated the expression of EphBRs and found them to be widely expressed by circulating cells as well as by bone-marrow-derived cells. Further expression-profiling experiments identified EphB2 and EphB4 as the preferentially and most-consistently expressed monocyte EphBRs. These findings confirm and extend previous studies demonstrating EphBR expression in platelets (Prevost et al., 2005), T-cells (Yu et al., 2003), dendritic cells (de Saint-Vis et al., 2003), bone-marrow hematopoietic cells (Okubo et al., 2006) and endothelial progenitor cells (Foubert et al., 2007).

Luminal ephrin-B2 expression and expression of EphBRs by monocytes provided the rationale for our analysis of the contribution of EphB4–ephrin-B2 adhesive interactions during monocyte recruitment. This hypothesis was also supported by recent observations associating EphB4 and ephrin-B2 with inflammatory processes (Yuan et al., 2004; Zamora et al., 2006). The subsequent experiments showed conclusively that EphB-expressing monocytes adhered preferentially to and transmigrate through ephrin-B2-expressing endothelium. This adhesion was dependent on the cytoplasmic domain of both EphB4 and ephrin-B2, which indicates distinct signaling mechanisms in ECs (ephrin-B2 reverse signaling) and in monocytes (EphB4 forward signaling). The mechanistic analysis of the bi-directional signaling processes during monocyte-to-EC adhesion is subject of future work. Surprisingly, preliminary data suggest that the PDZ domain of ephrin-B2 is not required for the preferential adhesion of monocytes to ephrin-B2-expressing ECs (data not shown). In contrast to the 30-minute cellular adhesion experiments, single cell atomic force spectroscopy (SCFS) detected adhesive forces resulting from a direct molecular interaction of the extracellular domains of ephrin-B2 with corresponding EphBRs in 1-minute short-term experiments. In these experiments, both EphB2 and truncated ephrin-B2 had a similar binding strength that was significantly higher than the binding strength of mock-transduced cells. The differences between the 1-minute force-measurement experiments and the 30-minute functional experiments most probably reflect the involvement of the cytoplasmic domains of receptor and ligand and might also affect adhesion strength by affecting intracellular signal-transduction pathways.

EphBR–ephrin-B2 interactions mediate transient and interactive cell-cell processes (Zimmer et al., 2003), and are therefore perfectly positioned to contribute to the dynamic adhesive mechanisms that occur during leukocyte recruitment. Future work will have to address in more mechanistic detail at which stage of the leukocyte recruitment cascade EphBR–ephrin-B2 interactions need to be positioned, and how they interact with the classical selectin, integrin and Ig-superfamily adhesion events. For example, EphB4 activation has recently been shown to upregulate PSGL-1 in endothelial

---

**Fig. 7.** Translocation of the EphBR–ephrinB complex to inter-endothelial junctions prior to internalization. HUVECs that overexpress full-length ephrin-B2 or ΔC-ephrin-B2 were stimulated for various periods of time with EphB4-Fc, after which monolayers were trypsinized, lysed and processed for western blot analysis. (A) Ephrin-B2-overexpressing HUVECs presented ephrin-B2 on the cell surface. Trypsin treatment removed surface-expressed ephrin-B2 (lane 2 vs lane 1). Internalized ephrin-B2 was detectable within 15 minutes of EphB4-Fc stimulation. Maximal internalization of the EphB4–ephrin-B2 complex was detected after 30-60 minutes. Internalized ephrin-B2 was eventually degraded, as evidenced by the absence of ephrin-B2 after 4 hours. (B) By contrast, ΔC-ephrin-B2, albeit being correctly expressed at the cell surface (B, lane 2 vs lane 1), was not endocytosed following EphB4-Fc stimulation; an unspecific band was detectable at the same size as ΔC-ephrin-B2 (arrow in B). Yet, this band was also detectable in full-length ephrin-B2 overexpressing cells (arrow in A). (C) HUVECs that overexpress full-length ephrin-B2 or ΔC-ephrin-B2 were stimulated with EphB4-Fc for various periods of time after which monolayers were fixed and stained for ephrin-B2. Unstimulated HUVECs overexpressed ephrin-B2 uniformly on their cell surface (0 min). Stimulation with EphB4-Fc for 20 minutes led to the translocation of the resulting EphB4–ephrinB2 and EphB4–ΔC-ephrin-B2 complex to inter-endothelial junctions (20 min). Complexes of full-length ephrin-B2 and EphB4-Fc were internalized within 60 minutes. By contrast, ΔC-ephrin-B2 complexes with EphB4-Fc were retained at inter-endothelial junctions (60 min). Analysis by fluorescence microscopy. (D) HUVECs that overexpress full-length ephrin-B2 or ΔC-ephrin-B2 were stimulated with EphB4-Fc for 30 minutes after which monolayer were fixed and stained for ephrin-B2 and VE-cadherin. Vascular integrity was not affected as evidenced by the unaltered junctional VE-cadherin expression. Ephrin-B2 did not colocalize with VE-cadherin; analysis by single scan confocal microscopy showing a single plain through the cell (notice the difference compared with images shown in C). Scale bars: 50 μm.
Journal of Cell Science

In summary, the interaction of EphBR-expressing hematopoietic cells with ephrin-B2-expressing ECs elicits pro-adhesive functions that lead to the translocation of EphBR–ephrin-B2 complexes to inter-endothelial junctions and also supports monocyte transmigration. Collectively, these findings suggest important roles of the EphBR–ephrinB system in controlling the recruitment of hematopoietic cells to the vascular endothelium. Further study of complementary EphBR–ephrinB expression patterns as well as the underlying signaling mechanisms will shed light into the leukocyte-adhesion cascade and will significantly expand our understanding of cell trafficking in the vasculature.

Materials and Methods

Primary cells, cell lines and media

BM cells were obtained by flushing femurs of C57BL/6 mice with HBSS, filtered through a 40 μm cell strainer (Becton Dickinson) and washed twice in cold phosphate-buffered saline. Mouse peripheral blood was collected and lymphocytes were obtained after sedimentation of erythrocytes in PBS, 5 mM EDTA, 2% dextran for 30 minutes at 37°C and further depleted of red blood cells by lysis, using a whole-blood erythrocyte lysing kit from R&D Systems as previously described (Reiss et al., 2003). Splenocytes were freshly isolated from disaggregated mouse spleens and cleared of red blood cells using a whole blood erythrocyte lysing kit (R&D Systems). Cells were incubated for 1 hour at 37°C in RPMI, then washed and resuspended to 1.5×10^6 cells/ml as described (Warneck et al., 2000). F4/80+ cells were isolated by rat anti-mouse F4/80 antibodies coupled to anti-rat magnetic beads and sorted according to the manufacturer’s instructions (MACS system, Miltenyi Biotek). Purity was determined by F4/80 FITC staining. PBMCN (peripheral blood mononuclear cells) from human blood were positively selected for monocytes using CD14+ magnetic beads according to the manufacturer’s protocol (MACS system, Miltenyi Biotek). Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany). HUVECs cultured from passages 4–8 were used for the experiments. EC growth medium (ECGM), EC basal medium (ECBM) and the corresponding medium supplements were purchased from Promocell (Heidelberg, Germany). The human monocyte cell line U937 was cultured in RPMI 1640 using Glutamax-1 (Invitrogen, Karlsruhe, Germany). The mouse macrophage cell line J774 and the human melanoma cell line A375 were grown in DMEM L-glutamine supplemented with 4.5 g/l glucose (Invitrogen, Karlsruhe, Germany). All cells were cultured at 37°C, 5% CO2, 100% humidity in the corresponding medium, containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (PAA, Pasching, Austria).

Antibodies and reagents

Recombinant mouse ephrin-B2-Fc and mouse EphB4-Fc, goat anti-ephrin-B2 antibody, goat anti-EphB2 antibody and goat anti-EphB4 antibody were obtained from R&D Systems (Wiesbaden, Germany). Goat IgG, human IgG (Fc-fragment) and mouse IgG were purchased from Jackson ImmunoResearch (Suffolk, UK). Goat anti-human actin antibody was obtained from Santa Cruz (Heidelberg, Germany). Mouse anti-human VE-cadherin antibody was purchased from Santa Cruz (Heidelberg, Germany). Rat anti-mouse F4/80 antibody was obtained from Serotec (Düsseldorf, Germany). As secondary antibody, goat anti-human IgG-Cy3 was obtained from Sigma-Aldrich (Taufkirchen, Germany), rabbit anti-goat HRP was obtained from Dako (Hamburg, Germany), rabbit anti-human IgG-FITC was obtained from PAA, Pasching, Austria. Mouse anti-human IgG-Alexa-Fluor-488 and donkey anti-goat IgG-Alexa-Fluor-488 were obtained from Invitrogen (Karlsruhe, Germany).

Transduction of HUVE and J774 cells

Full-length ephrin-B2 and EphB4 were cloned from freshly isolated HUVEC cDNA by using reverse transcriptase (RT)-PCR amplification, using specific primers. EphrinB2 constructs that lack the cytoplasmic domain were generated using primers that included the first 266 amino acid residues and lacked the terminal 67 amino acids (designated ΔC-ephrin-B2). EphB4 constructs that lack the cytoplasmic domain were generated using primers that only included the first 607 amino acid residues and lacked the terminal 380 amino acids (designated ΔC-EphB4). Sequence-controlled cDNAs were subcloned into a plbh-IRES-GFP-neo expression vector for retroviral transduction. HUVECs or J774 cells were retrovirally transduced following the instructions of a retroviral expression system (Clontech, Mountain View, CA). Individual clones resistant for G418 (PAA, Pasching, Austria) were isolated and expanded. The GFP-tag was used to monitor the efficacy of transduction via live imaging using a GFP filter on the fluorescent microscope (Olympus). Protein expression was confirmed by western blotting and corresponding staining procedures.

Proliferation assay

Transduced HUVECs were seeded into 96-well tissue culture plates (800 cells per well); proliferation assays were performed 24 hours, 48 hours and 72 hours after seeding using the Cell Proliferation Kit I (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Quantification was performed in triplicates.

Adhesion assay

Transduced HUVECs were grown to confluence in 24-well plates. U937 or J774 cells were pre-treated for 1 hour using anti-EphB4 and anti-EphB2 antibodies (2 μg/ml) or corresponding IgG control (2 μg/ml) for blocking experiments. J774 cells were pre-treated for 1 hour using anti-EphB4 and anti-EphB2 antibodies (2 μg/ml) or corresponding IgG control (2 μg/ml) for blocking experiments. Non-
adherent cells were washed off with PBS and the remaining adherent cells were fixed with 4% formaldehyde for 10 min. Quantification was performed with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin and the Cell-F software (Olympus) by counting the labelled cells at 4× magnification per high-power field (HPF). Quantification was performed in three wells counting six to eight fields per well.

For flow experiments, flow chamber μ-Slides I (ibidi, Martinsried, Germany) were coated with 0.2% gelatine for 30 minutes at 37°C. Flow chambers were washed twice with PBS, and 10^5 transduced HUVECs in 100 μl medium were plated into each chamber and grown to confluence. Subsequently, 2×10^5 J774 cells were passed over the monolayers with a shear rate of 50 per second. Non-adherent cells were washed off with PBS and the adherent cells were fixed with 4% PFA for 10 minutes. Quantification was performed as described above. Quantification was performed by counting 20 fields per flow chamber slide.

Transmigration assay
Transduced HUVECs (5×10^5) were grown to confluence on a transwell filter (8-μm pore size, Corning, Schiphol-Rijk, The Netherlands) in EGM for 48 hours. Confluence was checked using calcein staining, by applying 1 mM HEPES pH 7.4, 2% fetal bovine serum, 2 mM EDTA pH 7.4, 0.05% Tween-20 and incubated with primary antibody (2 μg/ml in 3% BSA supplemented with TBS-T) for 1 hour at room temperature. Following extensive washings, the membranes were incubated with peroxidase-coupled secondary antibody. Bound antibody was detected with ECL (Amersham Biosciences, Uppsala, Sweden) and exposure to Biomax MR films. All experiments are expressed as mean ± s.d.; differences between experimental groups were analyzed by Student’s t-test. Quantitative AFM experiments were analyzed by one way ANOVA analysis of variance. All groups were compared with each other using the all pairwise multiple comparison procedure (Holm-Sidak). *P<0.05 was considered statistically significant.

The authors acknowledge the Nikon Imaging Center at the University of Heidelberg for the use of the confocal microscope. This research was supported by the following grants from the Deutsche Forschungsgemeinschaft: SPP1190 ‘The tumor-vehicle interface’ (Au83/10-1) and SFB-TR23 ‘Vascular Differentiation and Remodeling’, projects A3 to H.G.A., C1 to Y .R., and C5. to T.K. and M.H. H.G.A. is supported by an endowed chair from the Aventis Foundation.

References


