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Original Article

TNF-α-mediated adhesion of monocytes to endothelial cells—The role of ephrinA1

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ABSTRACT

The ligand ephrin A1 is more often discussed to play a role in the development of the atherosclerotic plaque and in this context especially in the monocyte adhesion to endothelial cells. As tumor necrosis factor-α (TNF-α) is known to induce monocyte adhesion to endothelium and ephrin A1 expression, the present study focuses on the involvement of ephrin A1 in TNF-α-mediated monocyte adhesion.

The analysis of different members of the Eph/ephrin system in TNF-α-treated human umbilical vein endothelial cells (HUVEC) revealed that especially ephrinA1 was found to be highly regulated by TNF-α compared to other members of the Eph family. This effect is also present in arterial endothelial cells from the umbilical artery and from the coronary artery. This regulation is dependent on NFκB-activation as shown by the expression of a constitutive-active IκB-mutant. By using siRNA-mediated silencing and adenoviral overexpression of ephrinA1 in HUVEC, the involvement of ephrinA1 in the TNF-α triggered monocyte adhesion to endothelial cells could be demonstrated. In addition, these results could be verified by quantitative adhesion measurement using atomic force microscopy-based single-cell force spectroscopy and under flow conditions. Furthermore, this effect is mediated via the EphA4 receptor. EphrinA1 does not influence the mRNA or protein expression of the adhesion receptors VCAM-1 and ICAM-1 in endothelial cells. However, the surface presentation of these adhesion receptors is modulated in an ephrinA1-dependent manner.

In conclusion, these data demonstrate that ephrinA1 plays an important role in the TNF-α-mediated adhesion of monocytes to endothelial cells, which might be of great importance in the context of atherosclerosis.

1. Introduction

Atherosclerosis and especially its complications as ischemic heart disease and stroke are actually still the leading cause of death worldwide [1].

During the initiation of an atherosclerotic plaque, adhesion and subsequent diapedesis of monocytes into the subendothelial layer represent crucial events [2–4]. Monocyte adhesion to the vessel wall involves tethering and rolling processes. Whereas rolling is mediated by different E- and P-selectins [5], the subsequent firm adhesion is regulated by different monocyte integrins, which interact with adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) localized on the surface of endothelial cells [6,7]. The increased presentation of various adhesion molecules like VCAM-1, ICAM-1 or E-selectin on the endothelial surface is mediated by different proinflammatory cytokines like tumor necrosis factor-α (TNF-α) or Interleukin-1 (IL-1) [8–10].

TNF-α is one of the most important cytokine players in systemic inflammation and immune response [11,12]. TNF-α induces the adhesion of monocytes to the endothelium by upregulating the expression of different proadhesive genes like VCAM-1 or ICAM-1. This proadhesive regulation is mainly mediated via the activation of the transcription factor nuclear factor "kappa-light-chain-enhancer" of activated B-cells (NFκB) [13,14].

The Eph-receptors represent the largest subgroup of receptor tyrosine kinases. Both, the Eph-receptors and their cognate ephrin ligands are membrane-bound proteins. Therefore, a direct cell–cell contact is necessary for interaction. A characteristic of the Eph family is the ability of bidirectional-signaling, which transduces signaling in the direction of the receptor-expressing cell (forward-signaling) or of the ligand-expressing cell (reverse signaling) [15]. The Eph-receptors and their ephrin ligands are divided into A- and B-class, depending on their sequence homology and binding preferences.

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The impact of Eph/ephrin system in several processes like tumor biology or developmental processes (angiogenesis and axonal-guidance) is well known. In contrast, the influence of Eph/ephrin interactions in the pathophysiology of atherosclerosis plaque development is so far not well understood.

The ligand ephrinA1 (B-61 or LERK-1) was first described as a TNF-α inducible gene in HUVEC [16,17]. Similar effects are found after endothelial stimulation with lipopolysaccharide (LPS) or IL-1. EphrinA1 is a glycosylphosphatidylinositol-linked membrane bound protein and can also exist in a soluble form [18]. The interaction of ephrinA1 with one of its receptors EphA2 was shown to be involved in embryonic and adult angiogenesis [19–21]. Recent studies affirm the assumption that ephrinA1 plays a role in the pathophysiology of atherosclerotic plaque development. Recently, our group demonstrated that ephrinA1-mediated EphA4 activation induces monocyte adhesion to the endothelium via Rho signaling and subsequent stress fibre formation. This ephrinA1/EphA4-mediated effect on monocyte adhesion is independent from transcriptional activation of adhesion molecules [22]. In addition, Funk et al. described an ephrinA1/EphA2-mediated effect on monocyte adhesion by transcriptional activation of VCAM-1 [23]. Recently, the importance of EphA2 for atherosclerotic plaque development was demonstrated in an *in vivo* model [24]. These findings and the fact that ephrinA1 is upregulated in endothelial cells after treatment with TNF-α enforced speculations about a potential involvement of ephrinA1 in TNF-α-mediated increase of monocyte adhesion.

2. Materials and methods

2.1. Cell culture, siRNA transfection and adenoviral transduction

Human umbilical vein endothelial cells (HUVEC) and human umbilical artery endothelial cells (HUAEC) were isolated from anonymously acquired umbilical cords. HCAEC were a kind gift from Prof. T. Chavakis (Department of Clinical Pathobiochemistry, TU Dresden) and were originally purchased from Promocell. Isolation (HUVEC, HUAEC) and culturing of endothelial cells were done as described previously [25]. For stimulation experiments, endothelial cells were initially seeded at a density of 2.5×10^4 cells/cm^2 and allowed to adhere for 24 h. Afterward, the medium was changed to medium containing the stimulants (TNF-α or LPS) or with PBS (dilution: 1:1.000). THP-1 cells (human acute monocytic leukaemia cell line, ATCC, USA) were grown in RPMI 1640 medium (PAA, Pasching, Austria) supplemented with 10% FCS and 1% antibiotics/antimycotics (PAA). The medium was changed every 3 days. Subculturing of cells was done at a density of 1×10^6 cells/ml, and the cells were resuspended at a density of 2×10^5 cells/ml in a T175 flask with a total volume of 40 ml. Primary human monocytes were isolated by a two-step density-gradient centrifugation as previously described [22].

Transient siRNA transfection in HUVECs was done using the Turbofect-siRNA transfection reagent (Thermo Scientific, Waltham, MA, USA) as described previously [25]. The sequences of the used siRNAs are summarized in Table 1. The siRNAs were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Table 1
Overview of the used siRNAs.

Target	siRNA denotation	siRNA sequence (5′ to 3′)	
	siScrambled	Sense	AGGUAGUGUAAUCGCCUUGTT
		Antisense	CAAGGCGAUUACACUACCUTT
EphrinA1	si472	Sense	GGACACAGCUACUACUACATT
		Antisense	UGUAGUAGUAGCUGUGUCCTT
EphA2	si2181	Sense	GUACCUGGCCAACAUGAACTT
		Antisense	GUUCAUGUUGGCCAGGUACTT
EphA4	si2425	Sense	GCAAUUGCCUAUCGUAAAUTT
		Antisense	AUUUACGAUAGGCAAUUGCTT

Adenoviral transduction of HUVEC was done at a multiplicity of infection (m.o.i.) of 100. The virus-containing medium was removed after 24 h and the cells were cultivated for another 24 h before further proceeding.

2.2. Construction of replication-deficient Adenovirus

Construction of replication-deficient adenoviruses was done using the AdenoX-System (Clontech, Mountain View, CA, USA). The mutated and constitutively-active IκB-mutant was constructed as previously described [25]. Human ephrinA1 was amplified from oligo-dT reverse transcribed cDNA from HUVECs using the primers listed below that contain *Nhe*I or *Kpn*I restriction site (underlined sequences), respectively, at the 5′ end and were cloned into pShuttle vector as described previously [25,26]. Primers used for amplification of the human ephrinA1 gene: Forward: CCGCTAGC-CTATGGAGTTCCTCTGG Reverse: CCGGTACC-TGTG GCATACACCTTCA.

2.3. Real-time RT-PCR

The NucleoSpin®II Kit (Macherey-Nagel, Düren, Germany) was used to isolate total RNA according to the manufacturer's protocol. cDNA was synthesized with the Revert Aid™ H Minus First Strand Synthesis Kit (Thermo Scientific) from 1 to 5 μg total RNA with oligo-dT primers. cDNA from 25 ng total RNA was used for each PCR reaction. Primers (Eurofins MWG Operon) used are listed below. PCR was performed on a CFX96 real-time PCR System (BioRad, Hercules, CA) using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The PCR conditions for all primer sets were as follows: initial denaturation at 95 °C for 8 min followed by 45 amplification cycles, each consisting of 95 °C for 20 s, 58 °C for 45 s and 72 °C for 20 s with a final extension step at 72 °C for 2 min and a subsequent melting point analysis (Table 2).

2.4. Western blot

Western blot analysis was done as previously described [27]. The following antibodies and dilutions were used: polyclonal rabbit anti-ephrinA1 (Santa Cruz, Santa Cruz, USA), polyclonal rabbit anti-VCAM-1 (Santa Cruz), monoclonal mouse anti-ICAM-1 (Leica, Wetzlar, Germany), monoclonal mouse anti-β-actin (Santa Cruz) 1:1000, sheep anti-mouse-HRP (Amersham, Piscataway, USA) 1:10000 and goat-anti-rabbit-HRP (Santa Cruz) 1:2500. Intensities of protein bands were quantified by densitometry using "QuantityOne" software (BioRad). Equal loading of proteins was determined by detection of β-actin.

2.5. Determination of soluble ephrinA1 in cell culture supernatants

For the determination of soluble ephrinA1 in cell culture supernatants of TNF-α-treated endothelial cells, 2.0×10^5 HUVEC were seeded in one well of a 12-well cell culture plate. After 24 h, cells were treated with 0.5 ml medium containing 10 ng/ml TNF-α or PBS (control) for 4 or 24 h. After the treatment, cell culture supernatants were saved and centrifuged for 5 min at 250×*g* and 4 °C to deplete cell debris. The supernatants were collected and immediately frozen in liquid nitrogen and stored at -80 °C until the further analysis. The determination of ephrinA1 concentrations in the cell culture supernatants was done using a Human ephrinA1 ELISA (Bluegene, Shanghai, PR China) according to the manufacturer's protocol.

2.6. Nuclear protein extraction and NFkB activity ELISA

HUVECs were seeded on culture plates (6 cm ∅) and infected with adenoviruses (AdXlacZ, AdXmIκB). Forty-eight hours after infection, cells were treated with 10 ng/ml TNF-α or with PBS as a control for 2 h. After this treatment, cells were harvested and nuclear proteins