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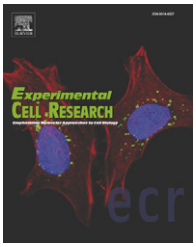

Research Article

Coxsackie- and adenovirus receptor (CAR) is expressed in lymphatic vessels in human skin and affects lymphatic endothelial cell function in vitro

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ABSTRACT

Lymphatic vessels play an important role in tissue fluid homeostasis, intestinal fat absorption and immunosurveillance. Furthermore, they are involved in pathologic conditions, such as tumor cell metastasis and chronic inflammation. In comparison to blood vessels, the molecular phenotype of lymphatic vessels is less well characterized. Performing comparative gene expression analysis we have recently found that coxsackie- and adenovirus receptor (CAR) is significantly more highly expressed in cultured human, skin-derived lymphatic endothelial cells (LECs), as compared to blood vascular endothelial cells. Here, we have confirmed these results at the protein level, using Western blot and FACS analysis. Immunofluorescence performed on human skin confirmed that CAR is expressed at detectable levels in lymphatic vessels, but not in blood vessels. To address the functional significance of CAR expression, we modulated CAR expression levels in cultured LECs in vitro by siRNA- and vector-based transfection approaches. Functional assays performed with the transfected cells revealed that CAR is involved in distinct cellular processes in LECs, such as cell adhesion, migration, tube formation and the control of vascular permeability. In contrast, no effect of CAR on LEC proliferation was observed. Overall, our data suggest that CAR stabilizes LEC–LEC interactions in the skin and may contribute to lymphatic vessel integrity.

Introduction

Our body is equipped with two vascular systems, namely, the blood vascular and the lymphatic system. While the main function of the circulatory blood vascular system consists in transporting oxygen and nutrients to the tissues, the open-ended lymphatic system takes up interstitial fluid that has leaked from the blood vessels into the tissue and returns it to the blood. Additionally, the lymphatic system is involved in the absorption of dietary fats in the intestine, as well as in immune surveillance by leukocytes that migrate through lymphatic vessels [1,2]. Similarly to the blood vascular systems, also the lymphatic system actively participates in pathologic conditions, such as in chronic inflammation, tumor growth and metastasis — processes that are characterized by substantial vascular remodeling, i.e. (lymph) angiogenesis [3–5]. Due to the lack of suitable lymphatic-specific markers, lymphangiogenesis and other aspects of lymphatic vessel biology have for a long time only been marginally studied.

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However, during the last decade, lymphatic markers such as Prox-1 [6], LYVE-1 [7] and podoplanin [8] have been identified and now make it possible to better investigate lymphatic vessel biology and function in vitro and in vivo [9].

Coxsackie- and adenovirus receptor (CAR) was originally identified as the primary attachment protein for the entry of coxsackie B and adenoviruses into cells [10]. CAR is a 46-kDa transmembrane glycoprotein and belongs to the CTX-subfamily of immunoglobulin-like surface molecules. Similar to other members of this family, such as junctional adhesion molecules (JAMs), or endothelial cell-selective adhesion molecule (ESAM), CAR appears to mainly function in the context of cell adhesion [11,12]. However, also an involvement of CAR in the control of cell proliferation has recently been reported [13–16]. CAR has been shown to engage into homophilic interactions [12], but also heterophilic interactions of CAR with other ligands have been reported [17]. Although considerable knowledge exists on the function of CAR as a viral receptor, the physiological role and expression pattern of CAR in human tissues is less studied. CAR expression greatly varies between cell types and tissues during embryonic development and adulthood [12]. While CAR is strongly expressed in the developing hearts and brain of mice [18–20] its expression during adulthood is largely confined to epithelial cells, such as in the trachea, in the intestine or in the skin [21]. Several studies have also reported on an altered expression pattern of CAR during pathologic conditions: For example, CAR becomes expressed on various malignant cells and, consequently, has been intensively studied as a potential target receptor for adenovirus-based gene therapies [22]. Interestingly, several studies have shown an inverse correlation between CAR expression and tumor progression, likely due to CAR-mediated suppression of tumor cell proliferation and migration [13,23,24].

Tight junctions are not only a characteristic feature of epithelial cells, but also of endothelial cells. Similarly to epithelial cells, endothelial cells function as gatekeepers and control the passage of cells and fluids to and from tissues. In fact, several tight junction proteins, such as JAMs, CD99, ESAM or the adaptor proteins claudin and occludin are expressed in the vasculature [25]. By contrast, contradicting results exist on the expression of CAR in endothelial cells. Several studies have reported on the expression of CAR in cultured human endothelial cells [26–28], but other studies found no expression of CAR in endothelial cells in various murine tissues [18,21] or in the vasculature of human heart tissue [29]. A detailed analysis of CAR expression in human tissues, in combination with vascular markers has, however, not been performed to date.

In search of further lymphatic endothelial cell-specific markers, we have recently performed microarray analyses of cultured blood vascular and lymphatic endothelial cells isolated from human skin. Comparison of the gene expression profile of these two cell types revealed that CAR was on average 15-fold higher expressed on skin-derived lymphatic endothelial cells (LECs) than on blood vascular endothelial cells (BECs) at the mRNA level. In the present study, we have first validated these data by quantitative RT-PCR. Using Western blot and FACS analysis, higher expression levels of CAR could also be confirmed at the protein level in cultured human LECs, as compared to human umbilical vein endothelial cells (HUVECs). Immunofluorescence performed on human skin sections detected CAR on lymphatic but not on blood vascular endothelial cells. To address the functional role of CAR in LECs, we used siRNA technology and CAR expression vectors to modulate the expression of CAR protein in cultured human LECs in vitro. Functional assays performed with the transfected cells revealed that LEC-expressed CAR is involved in distinct aspects of LEC biology, such as in LEC–LEC adhesion, migration, tube formation and vascular permeability. Overall, our data suggest that CAR may contribute to LEC-mediated processes and lymphatic vessel stability in human tissues such as the skin.

Materials and methods

Cells and reagents

Human LECs were isolated from neonatal human foreskins and characterized as previously described [30]. Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex (Verviers, Belgium) and ScienCell (Carlsbad, USA). Cells were cultured in Endothelial Basal Medium (EBM; Cambrex) supplemented with 20% fetal bovine serum (FBS) (GIBCO, Paisley, UK), antibiotic antimycotic solution (1×; Fluka, Buchs, Switzerland), L-glutamine (2 mM; Fluka), Hydrocortisone (10 μg/ml; Fluka) and N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (cAMP, 2.5×10^{-2} mg/ml; Fluka), unless otherwise noted. Cells were grown at 37 °C in 5% CO_2 and cultured for up to ten passages. TNFα used for in vitro assays was obtained from R&D Systems (Abingdon, U.K.).

Quantitative real-time PCR

Total cellular RNA was extracted from confluent plates of LECs or HUVECs using TRIZOL® reagent (Invitrogen, Paisley, UK) and treated with RQ1 RNase-free DNase I (Promega, Dübendorf, Switzerland) and RNase-Inhibitor (Applied Biosystems, Rotkreuz, Switzerland) following the manufacturers' protocol. The expression levels of CAR were examined by real-time PCR on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The following 3 sets of primers (1 μM final concentration) were used for the comparison of CAR mRNA levels in LECs and HUVECs: CXADR.1 forward cgactctcccacctgctaag and reverse tgctctgtgctggaatcatc (5′ to 3′); CXADR.2 forward cagtgcctgttgcgtctaaa and reverse gcgctagagcaagcaaagtt; CXADR.3 forward cctgtgcggagtagtggatt and reverse gtttccccttggctttttc (all from Sigma-Aldrich, Buchs, Switzerland). mRNA levels of RNA polymerase II (i.e. housekeeping gene) were determined for normalization using the QuantiTect Primer Assay from Qiagen (Polr2a; QT00033264, Hs_POLR2A_1_SG; Qiagen, Hombrechtikon, Switzerland). Reverse transcription was performed for 30 min at 48 °C, with 25 U/reaction of Multiscribe RT (Applied Biosystems). For the subsequent qPCR reaction SYBR Green PCR Master Mix (Applied Biosystems) was used. To determine the effect of TNFα treatment on CAR mRNA expression in human LECs, TaqMan Gene Expression Assays were performed using TaqMan One-Step RT-PCR Master Mix (Applied Biosystems) and the following primers and probes: human LYVE-1 (forward AGCTATGGCTGGGTTGGAGA, reverse CCCCATTTTTCCCACACTT, probe FAM-TTCGTGGTCATCTCTAGGAT-TAGCCCAAACC-BKH1); human ICAM-1 (Inventoried TaqMan Gene Expression Assay, Hs99999152_m1, Applied Biosystems), β-actin (forward TCACCGAGCGCGGCT, reverse TAATGTCACGCACGATTTCCC, probe JOE-CAGCTTCACCACCACGGCCGAG-BKH1); All qRT-PCR reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems).