Journal of Dermatological Science 71 (2013) 184–194

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Activation of the epidermal growth factor receptor promotes lymphangiogenesis in the skin

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ARTICLE INFO

Article history:
Received 28 August 2012
Received in revised form 21 April 2013
Accepted 23 April 2013

Keywords:
Angiogenesis
Lymphangiogenesis
EGF
EGF receptor

ABSTRACT

Background: The lymphatic vascular system regulates tissue fluid homeostasis and plays important roles in immune surveillance, inflammation and cancer metastasis. However, the molecular mechanisms involved in the regulation of lymphangiogenesis remain incompletely characterized.
Objective: We aimed to identify new pathways involved in the promotion of skin lymphangiogenesis.
Methods: We used a mouse embryonic stem cell-derived embryoid body vascular differentiation assay to investigate the effects of a selection of pharmacological agents with the potential to inhibit blood and/or lymphatic vessel formation. We also used a subcutaneous Matrigel assay to study candidate lymphangiogenesis factors as well as skin-specific transgenic mice.
Results: We found that compounds inhibiting the epidermal growth factor (EGF) receptor (EGFR) led to an impaired formation of lymphatic vessel-like structures. *In vitro* studies with human dermal lymphatic endothelial cells (LECs), that were found to express EGFR, revealed that EGF promotes lymphatic vessel formation. This effect was inhibited by EGFR-blocking antibodies and by low molecular weight inhibitors of the EGFR associated tyrosine kinase. Incorporation of EGF into a mouse matrigel plug assay showed that EGF promotes enlargement of lymphatic vessels in the skin *in vivo*. Moreover, transgenic mice with skin-specific overexpression of amphiregulin, another agonistic ligand of the EGFR, displayed an enhanced size and density of lymphatic vessels in the skin.
Conclusion: These findings reveal that EGFR activation is involved in lymphatic remodeling and suggest that specific EGFR antagonists might be used to inhibit pathological lymphangiogenesis.

1. Introduction

The lymphatic vascular system plays an essential role in physiological fluid homeostasis. It is also involved in several pathological conditions, including inflammation and cancer metastasis [1]. In recent years, our understanding of how lymphatic endothelial cell (LEC) differentiation, growth and function are regulated has significantly increased [1]. This progress became possible based on the discovery of lymphatic endothelium-specific markers, namely podoplanin [2] and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) [3], and on studies of lymphatic system development in genetic mouse models [1].

Vascular endothelial growth factor-C (VEGF-C) is the best characterized lymphangiogenic factor and predominantly activates VEGF receptor (VEGFR)-3. Under normal conditions, VEGFR-3 is expressed by LECs but not by the endothelial cells of blood vessels. Activation of VEGFR-3 promotes LEC proliferation and migration *in vitro* [4] and lymphatic vessel formation *in vivo* [5]. Lymphangiogenesis is also stimulated by VEGF-A [6–8]. Additional growth factors including fibroblast growth factor-2, hepatocyte growth factor, angiopoietin-1 and -2, and platelet-derived growth factor, have been shown to promote lymphangiogenic processes [9]. Because of the emerging role of the lymphatic vascular system in human diseases such as cancer metastasis, chronic inflammation, organ transplant rejection and hypertension [1], understanding and modulating lymphangiogenesis is of primary interest. The present study was aimed at unraveling novel mechanisms involved in the regulation of lymphatic vessel formation.

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0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2013.04.024

2. Materials and methods

2.1. Mouse embryonic stem cell culture, establishment and treatment of embryoid bodies (EBs)

Murine C57BL/6x129SvEv derived embryonic stem cells (mES cells; passage 3–12; kindly provided by N. Gale, Regeneron Pharmaceuticals, Tarrytown, NY, USA), were cultured on mitotically inactivated primary mouse embryonic fibroblasts (PMEFs, passage 2–5, Institute of Laboratory Animal Science, University of Zurich, Switzerland) in Dulbecco's modified Eagle medium (Gibco, Eggenstein, Germany), supplemented with 18% fetal bovine serum (FBS; Gibco), 100 nM sodium pyruvate (Sigma–Aldrich, Buchs, Switzerland), MEM vitamins, 2 mM L-glutamine, streptomycin and penicillin (all from Gibco), 10 mM 2-mercaptoethanol and 2000 U/ml recombinant leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA, USA). PMEFs and LIF were removed and mES cells were transferred to suspension culture for embryoid body (EB) formation as described [10,11]. After 3 or 4 days, EBs of similar size were transferred into 12-well dishes (BD Bioscience, San Diego, CA, USA). This step is termed "initiation of the EBs" throughout the text. The EBs were cultured for 14 days and then incubated for 4 days with either 100 ng/ml human recombinant epidermal growth factor (EGF, BD Biosciences) or a mixture of 10 μM all-trans-retinoic acid (RA; Sigma–Aldrich), 0.5 mM 3′,5′-cyclic monophosphate (cAMP; Fluka, Buchs, Switzerland), and 200 ng/ml recombinant human VEGF-C (R&D Systems, Minneapolis, MN, USA). These agents were used alone or in combination with one of the following pharmacological agents (all from Sigma–Aldrich) added at 10 μM concentrations: 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (genistein); N4-(1-benzyl-1H-indazol-5-yl)-N6,N6-dimethyl-pyrido[3,4-d]pyrimidine-4,6-diamine (GW2974); 3-(2,4-dimethylpyrrol-5-yl)-methylidene-indolin-2-one (SU5416). Medium only and medium containing 0.1% dimethyl sulfoxide (DMSO) were used as negative vehicle controls. EBs were fixed in 20 °C cold methanol for 10 min prior to analysis.

2.2. Immunofluorescence analysis of vessel development in EBs

EBs ($n = 9$ per group) were stained with antibodies against mouse LYVE-1 (Angiobio, Del Mar, CA and R&D Systems), CD31 (BD Bioscience), and secondary antibodies labeled with Alexa-Fluor 488 or 594 (Molecular Probes, Eugene, OR, USA) as described [11]. Cell nuclei were stained with Hoechst bisbenzimide (Sigma–Aldrich). The stained samples were examined with a Zeiss Axiovert 200 M microscope, images were captured with a Zeiss AxioCam-MRm (Carl Zeiss; Oberkochen, Germany) and the Axio Vision4.4 software (Zeiss). Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA) was used for image overlay. Computer-assisted morphometric vessel analyses were performed using the IP-LAB software (Scanalytics; Fairfax, VA, USA). The lymphatic vessel area was determined relative to the total EB area. The vessel number per EB was quantified by manually counting all independent CD31+/LYVE-1+ structures present in an EB. Statistical analysis (unpaired Student's *t*-test) was performed using Microsoft Excel 2003.

2.3. Proliferation, migration and tube formation of human lymphatic endothelial cells

Human dermal microvascular LECs were isolated from neonatal human foreskins by immunomagnetic purification as described [12]. Cells were cultured in endothelial basal medium (Cambrex, Verviers, Belgium) with 20% FBS, antibiotic antimycotic solution, L-glutamine (2 mmol/L), hydrocortisone (10 μg/ml), and N^6,2′-O-dibutyryladenosine-3′,5′-cyclicmonophosphate sodium salt (25 μg/ml; all from Fluka, Buchs, Switzerland) at 37 °C and 5% CO_2 for up to 11 passages.

Cells were incubated with either 10 μM GW2974 (Sigma–Aldrich) or a vehicle control containing the same amount of DMSO. Recombinant human VEGF-A165 (20 ng/ml; kindly provided by the National Cancer Institute, Bethesda, MD, USA), or *Pichia pastoris*-derived human VEGF-C (1.2 μg/ml; kindly provided by Dr. K. Ballmer-Hofer, Paul Scherrer Institute, Switzerland) were used as positive controls. The relatively high amounts of VEGF-C used are based on the fact that N-glycans added by *P. pastoris* to proteins are of the oligomannose-type. Such glycans are bound by the mannose receptor expressed on lymphatic endothelial cells, likely leading to scavenging and endocytosis of VEGF-C and thus a lower availability of VEGF-C for binding to its receptor. In additional experiments, LECs were treated with 100 ng/ml human EGF with or without 10 μg/ml of recombinant humanized monoclonal antibody 2C4 (Pertuzumab, kindly provided by Roche Diagnostics GmbH, Mannheim, Germany), an EGFR blocking antibody (Calbiochem, Darmstadt, Germany) or mouse IgG (Santa Cruz Biotechnology, Heidelberg, Germany).

Proliferation, migration, and tube formation assays were performed as previously described [12]. For proliferation assays, LECs ($1.25–1.5 \times 10^3$) were seeded into fibronectin (Chemicon International, Temecula, CA, USA)-coated 96-well plates. After 72 h incubation with the agents mentioned above, cells were incubated with 5-methylumbelliferylheptanoate (MUH, Sigma–Aldrich) as described [13]. The intensity of fluorescence, which is proportional to the number of viable cells, was measured using a SpectraMax-Gemini EM microplate reader (Bucher Biotec AG, Basel, Switzerland). Ten wells per incubation mixture were analyzed.

For migration assays, two parallel lines and one orthogonal line were scratched into confluent LEC cultures, removing LECs without damaging the fibronectin coating. Pictures of the two crosses per well were taken immediately after scratching (T0) and after 16 h of incubation (T16) with a Zeiss AxioCam-MRm. The quantitative analysis of the non-closed area was performed using Photoshop C3. Three wells per incubation mixture were analyzed.

For tube formation assays, confluent LEC monolayers were overlaid with collagen type I gels (1 mg/ml; Cohesion, Palo Alto, CA, USA), that contained the studied compounds, as described [12]. Tube-like structure formation was evaluated for up to 20 h. Three representative pictures were taken per well, and tube length was analyzed using the IP Lab software as described [12]. Three wells per incubation mixture were used. For all these assays, three independent experiments were performed. Statistical analyses were performed using the two-tailed unpaired Student's *t*-test.

2.4. Immunoblotting

Confluent cultures of LECs were homogenized in lysis buffer [12]. Protein concentrations were determined using the NanoOrange Protein Quantification Kit (Molecular Probes, Eugene, OR, USA). The lysates (100 μg of total protein) were subjected to SDS-polyacrylamide gel electrophoresis using NuPAGE 10% BT gels, 1.0 mm, 12 wells. The proteins were transferred onto Trans-Blot Transfer Medium pure nitrocellulose membranes (BioRad, Hercules, CA, USA). A rabbit anti-human EGFR antibody (Abcam) and the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) were used for immunostaining. Equal loading was confirmed using an antibody against β-actin (Sigma).

2.5. Quantitative real-time PCR

A total of 1 μg of RNA isolated from human dermal microvascular LECs was used to generate cDNA with the high