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Original article

Cancer therapy modulates VEGF signaling and viability in adult rat cardiac microvascular endothelial cells and cardiomyocytes

Manuel Chiusa [a], Sara-Lynn Hool [a], Petra Truetsch [a], Siamak Djafarzadeh [b], Stephan M. Jakob [b], Franziska Seifriz [a], Stefan J. Scherer [c], Thomas M. Suter [a], Christian Zuppinger [a,*], Stephan Zbinden [a]

[a] Bern University Hospital, Cardiology, CH-3010 Bern, Switzerland
[b] Department of Intensive Care Medicine, Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland
[c] Hoffmann-La Roche, BioMarkers program, Switzerland

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ABSTRACT

This work was motivated by the incomplete characterization of the role of vascular endothelial growth factor-A (VEGF-A) in the stressed heart in consideration of upcoming cancer treatment options challenging the natural VEGF balance in the myocardium. We tested, if the cytotoxic cancer therapy doxorubicin (Doxo) or the anti-angiogenic therapy sunitinib alters viability and VEGF signaling in primary cardiac microvascular endothelial cells (CMEC) and adult rat ventricular myocytes (ARVM). ARVM were isolated and cultured in serum-free medium. CMEC were isolated from the left ventricle and used in the second passage. Viability was measured by LDH-release and by MTT-assay, cellular respiration by high-resolution oxymetry. VEGF-A release was measured using a rat specific VEGF-A ELISA-kit. CMEC were characterized by marker proteins including CD31, von Willebrand factor, smooth muscle actin and desmin. Both Doxo and sunitinib led to a dose-dependent reduction of cell viability. Sunitinib treatment caused a significant reduction of complex I and II-dependent respiration in cardiomyocytes and the loss of mitochondrial membrane potential in CMEC. Endothelial cells up-regulated VEGF-A release after peroxide or Doxo treatment. Doxo induced HIF-1α stabilization and upregulation at clinically relevant concentrations of the cancer therapy. VEGF-A release was abrogated by the inhibition of the Erk1/2 or the MAPKp38 pathway. ARVM did not answer to Doxo-induced stress conditions by the release of VEGF-A as observed in CMEC. VEGF receptor 2 amounts were reduced by Doxo and by sunitinib in a dose-dependent manner in both CMEC and ARVM. In conclusion, these data suggest that cancer therapy with anthracyclines modulates VEGF-A release and its cellular receptors in CMEC and ARVM, and therefore alters paracrine signaling in the myocardium.

1. Introduction

Cardiac microvascular endothelial cells (CMEC) play a critical role in cardiac development, contractile performance, and rhythmicity. Because of the high density of capillaries in the ventricular wall (intercapillary distance 20.2 μm) and the small (<1 μm) endothelial-cardiomyocyte distance, factors released by CMEC can modulate cardiac function and survival in the adult heart [1]. Besides serving as a barrier and facilitating the exchange of oxygen, the microvascular endothelium is also capable of secreting factors that support cardiomyocyte compensatory response to hemodynamic and oxidative stress. An important peptide factor essential for the function of endothelial cells under normal and pathological conditions is vascular endothelial growth factor (VEGF) and especially the founding member of the VEGF-family, VEGF-A. VEGF-A is constitutively expressed in several types of normal adult epithelium, endothelium, in macrophages, and cardiac myocytes [2,3]. It is thought to be one of the prime mediators of physiologic angiogenesis and it is strongly expressed in the vast majority of solid human and animal carcinomas leading to pathological angiogenesis [4]. VEGF family members bind to three high-affinity receptor tyrosine kinases, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (FLT-4) [4]. VEGFR-1 and VEGFR-2 are widely expressed by normal vascular endothelium in vivo and also by cultured endothelial cells. VEGFR-2 is generally expressed in higher copy numbers than VEGFR-1, but has a lower affinity for VEGF-A [5].

Cancer therapy with sunitinib, a multitargeted tyrosine kinase inhibitor acting on VEGFR's, stem cell factor receptor factor, CD 117 (c-KIT), platelet- derived growth-factor receptors (PDGF), FMS-like TK 3 (FLT3) and colony stimulating factor-1 (CSF-1) receptor, has been approved for clinical use in patients for renal cell carcinoma and other types of cancer [6,7]. Cardiovascular adverse effects such as hypertension, a reduction of left ventricular ejection fraction and congestive heart failure have been described [8–10]. Anthracyclines

* Corresponding author at: Cardiology, Bern University Hospital, Department of Clinical Research MEM E808, Murtenstrasse 35, CH-3010 Bern, Switzerland, Tel.: +41 31 6329143; fax: +41 31 6328837.
E-mail address: christian.zuppinger@dkf.unibe.ch (C. Zuppinger).

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are still widely used for cancer treatment, despite their cumulative cardiotoxicity, which is often irreversible, and can lead to congestive heart failure [11]. Proposed mechanisms of anthracycline-induced cardiotoxicity are induction of apoptosis, disruption of energy production, myofibrillar damage, and formation of reactive oxygen species (ROS) [12]. Despite the substantial amount of literature on anthracycline-associated cardiac adverse effects, little is known about effects on the microvascular endothelium and paracrine signaling during cancer therapy. To the best of our knowledge, the current study is the first to investigate cardiotoxicity and anthracycline-associated effects on VEGF production in CMEC.

2. Materials and methods

2.1. Adult rat ventricular myocytes (ARVM) isolation

Adult (250–350 g) male Wistar rats from an in-house breeding facility were anesthetized by pentobarbital (Esconarkon®, Streuli Pharma AG, Uznach, Switzerland) injection. Isolation of calcium-tolerant cardiomyocytes was achieved according to previously published methods [13]. The cardiomyocytes were plated onto laminin-coated coverslips (mouse laminin, Invitrogen, LuBioScience, Lucerne, Switzerland) and maintained for the entire culture period in serum-free ACCT-medium containing: fatty acid-free bovine serum albumin (BSA) 2 mg/mL, L-carnitine 2 mmol/L, creatine 5 mmol/L, taurine 5 mmol/L, triiodothyronine 10 nM (all from Sigma, Buchs, Switzerland), penicillin 100 IU/ml, and streptomycin 100 mg/ml (Invitrogen) in MEM199 (Amimed, BioConcept, Allschwil, Switzerland). All experiments were carried out according to the Swiss animal protection law and with the permission of the state veterinary office. This investigation conforms to "the Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2. Isolation and culture of cardiac microvascular endothelial cells (CMEC)

Briefly, cardiac microvascular endothelial cells were enzymatically released from left ventricular tissue of adult (250–350 g) male Wistar rats using 0.2% collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA) in sterile Hank's buffer solution (HBSS)(Invitrogen, LuBioScience, Lucerne, Switzerland) for 20 min at 37 °C followed by 0.25% trypsin (Invitrogen) for 20 min at 37 °C in a shaking water bath according to the method developed by Nishida et al. [14] with modifications. CMEC reached confluence within 3–4 days in DMEM (Amimed, BioConcept, Allschwil, Switzerland) with 20% newborn calf serum (NCS) (Invitrogen) and antibiotics. No additional growth factors were added to the growth medium. CMEC were then used in the second passage and starved for one day in DMEM with 1% NCS before pharmacologic treatments. CMEC were characterized by immunostaining for cellular markers according to Ando et al. [15] (Fig. 1).

2.3. Western blot analysis

ARVM and CMEC were maintained in serum-free medium or in DMEM with 1% NCS respectively, and treated as indicated in the results section. ARVM and CMEC were quickly washed with ice-cold phosphate buffered saline and lysed using modified RIPA lysis buffer (1% NP-40, 0.25% deoxycholic acid, 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 2 μg/ml pepstatin, 1 μg/ml aprotinin) (Sigma, Buchs, Switzerland). Cell lysates were boiled with a loading buffer containing 3.3% glycerin, 1% SDS 20 mM TRIS pH 6.8, 23 mM β-mercaptoethanol freshly added, and 0.4 mg/ml bromophenolblue). Proteins were separated by SDS-PAGE using precast 4–20% gradient gels (Lonza, LuBioScience, Lucerne, Switzerland) and blotted to Protran nitrocellulose (Whatman, Dassel, Germany) or polyvinylidenfluorid (PDVF)(Millipore, Zug, Switzerland) membranes with low autofluorescence (Millipore). Immunodetection was carried out after blocking in 5% milk or gelatin in TTBS (20 mM Tris base, pH 7.5, 150 mM NaCl, 0.05% Tween-20) and followed by incubation with diluted specific antibodies.

2.4. Antibodies used for immunofluorescence microscopy and Western blotting

Rabbit monoclonal antibodies to AKT1 (phospho-Ser473) were purchased from Cell Signaling (BioConcept, Allschwil, Switzerland), monoclonal antibodies (mAb) to pERK1/2 (phospho-Tyr204) from Santa Cruz Biotech (LabForce, Nunningen, Switzerland) (sc154). Rabbit polyclonal antibodies (pAb) to HIF-1α (cat nos. 100–449) were obtained from Novus biologicals (LuBio Science, Lucerne, Switzerland). pAb to all-actin (A2066, Sigma), mAb to CD31 (MCA1334G, AbD Serotec) (Morphosys AbD, Düsseldorf, Germany), pAb to von Willebrand factor (A0082, Dako) (Dako Schweiz, Baar, Switzerland), mAb to desmin (D1033, Sigma) and vimentin (Dako, clone V9) were used for the characterization of CMEC. Mab to sarcomeric actin (A2172, Sigma), all-actin (A2066, Sigma), pAb to total Erk1/2 (06-182, Milipore) and mAb to alpha smooth muscle actin (SMA) (A2547, Sigma), were used as a reference for normalizing the protein amount in each lane for western blotting. For the visualization of signals, a LiCor Odyssey infrared imaging system was used with secondary antibodies coupled to IRDye fluorescent dyes (LI-COR Biotechnology, Bad Homburg, Germany). Secondary antibodies coupled to Alexa fluorescent dyes (Invitrogen) were used for immunofluorescence staining.

2.5. Growth factor and pharmacologic treatments

Recombinant VEGF-A (rat VEGF-A_{165}, cat no 400–31) and VEGF-B (human VEGF-B_{167}, cat no 100-20B) were purchased from Peprotech (PeproTech EC, London, UK). Recombinant human insulin-like growth factor-I (IGF-I) was from Sigma. We have used rat-specific recombinant proteins as much as possible. For IGF-I a strong activation of Akt was found in ARVM thereby providing a positive control for this pathway (supplemental Fig. 2). Kinase inhibitors SB203580 (cat. no. 559389) and U0126 (cat. no. 662005) were from EMD4Biosciences (Merck, Darmstadt, Germany). Cobalt chloride ($CoCl_2$), N-acetylcystein (NAC) and phorbol-12-myristate-13-acetate (PMA) were from Sigma.

2.6. VEGF-A ELISA

VEGF-A quantification by sandwich ELISA was performed according to the manufacturer (rat VEGF-A ELISA kit, R&D Systems Europe, Abingdon, UK). Results were normalized against untreated CMEC or ARVM and absorption was measured using a Safire microplate reader (Tecan group, Männedorf, Switzerland).

2.7. MTT and WST-8 viability assay

The reduction of tetrazolium salts in the MTT and WST-8 assay is generally considered an indicator of cellular viability and was shown to be a measure of the rate of glycolytic NADH production by microsomal enzymes [16]. CMEC or ARVM were washed and incubated for 2 h at 37 °C in the dark with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) solved in Tyrode's solution or in cell culture medium. CMEC or ARVM were then washed and lysed to release formazan (lysis buffer: 0.6% glacial acetic acid and 10% SDS in DMSO) and lysates were analyzed at a Safire multiwell-plate reader (Tecan). Results were corrected for cell density and normalized for the control condition. For the WST-8 assay