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Stanniocalcin-1 and -2 promote angiogenic sprouting in HUVECs via VEGF/VEGFR2 and angiopoietin signaling pathways

Alice Y.S. Law, Chris K.C. Wong *

Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong

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ABSTRACT

The members of stanniocalcins (STCs: STC-1 and STC-2) family are known to be involved in tumor progression and metastasis. Although current evidences suggest the involvement of STCs in vascular biology, the functional roles of STCs in angiogenesis have not yet been elucidated. The objective of this study was to decipher the roles of STCs in angiogenesis of human umbilical vascular endothelial cells (HUVECs). We prepared STC1 or STC2 lentiviral particles to transduce the cells to reveal their effects on the processes of cell proliferation, migration and tube formation. The stimulatory effects of STCs on these processes were demonstrated, supporting the notion of STCs in angiogenesis. To dissect the molecular components involved, STC1 or STC2 transduction led to significant increases in the expression levels of cell cycle regulators (i.e. cyclin-D and phospho-retinoblastoma), matrix metalloproteinase (MMP)-2 but a decrease of tissue inhibitors of metalloproteases (TIMP)-1. The expression levels of the cell adhesion/junctional proteins vimentin and VE-cadherin, were significantly induced. Moreover the transduction induced both mRNA and protein levels of eNOS, VEGF and VEGFR2 (KDR mRNA and pKDR), highlighting the stimulatory effects of STCs on VEGF-signaling pathway. Furthermore STC2 transduction but not STC1, activated angiopoietin (Ang)-2 pathway. Taken together, STC1 and STC2 play positive roles in angiogenic sprouting. The action of STC1 was mediated via VEGF/VEGFR2 pathway while STC2 were mediated via VEGF/VEGFR2 and Ang-2 pathways.

1. Introduction

Stanniocalcin-1 (STC1) is a hypocalcemic glycoprotein hormone secreted from the endocrine gland, corpuscle of Stannius to regulate Ca^{2+} and Pi homeostasis in bony fish. The mammalian homologs of stanniocalcins (STC1 and STC2) are known to be widely expressed in various human tissues and to act in paracrine/autocrine manner. Their expression levels are also found to be regulated in diverse physiological (i.e. renal Pi reabsorption, wound healing) or pathological conditions (i.e. inflammation and carcinogenesis), indicating their involvement in some fundamental cellular processes (Yeung et al., 2012). In human cancer study, considerable numbers of reports have highlighted the involvement of STCs in tumor progression and metastasis. Our previous studies have demonstrated the direct transactivation role of HIF-1 on STC-1 or STC-2 expression (Law et al., 2010; Law and Wong, 2010a). The biological functions (i.e. epithelial mesenchymal transition (EMT), pro-/anti-apoptosis, proliferation) of their expression in hypoxic tumors were reported (Law et al., 2008b; Law and Wong, 2010a, 2010b). Other transcriptional factors BRCA1, Sp1, p53 or ATF4 known to regulate tumor progression, positively regulated STC-1 or STC-2 expression (Ching et al., 2012; Ito et al., 2004; Lai et al., 2007; Law et al., 2011; Welcsh et al., 2002). Generally the biological roles of STCs in tumor progression have been suggested.

Locally advanced solid tumors mostly suffer from hypoxia owing to rapid tumor growth and vascular insufficiency. To support cell survival and further progression of tumors, the onset of hypoxia-stimulated angiogenesis is one of the key factors to induce EMT, invasion and metastasis. Correlation studies have suggested that STC-1 was a vascular-specific angiogenesis-associated gene in colon tumors (Gerritsen et al., 2002) and was the downstream target of VEGF (Bell et al., 2001; Holmes and Zachary, 2008; Jauhiainen et al., 2011; Klein et al., 2009; Wary et al., 2003; Zlot et al., 2003). Similarly STC-2 protein was found to be localized in tumor vasculature of ovarian tumors (Buckanovich et al., 2007). High level of STC-2 expression was also positively correlated with venous invasion in gastric cancers (Yokobori et al., 2010). In a Boyden chamber assay, STC-2 enriched conditioned medium induced endothelial invasion of HUVEC (Law and Wong, 2010b). Although the possible involvement of STC-1 and STC-2 in angiogenesis has been suggested, the empirical mechanisms of action of STCs in new blood vessel formation are not known.

* Corresponding author. Tel.: +852 3411 7053; fax: +852 3411 5995.
E-mail address: ckcwong@hkbu.edu.hk (C.K.C. Wong).

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In the present study, we aimed to reveal the regulation and function of STC-1 and STC-2 in angiogenesis. Human umbilical vein endothelial cells (HUVEC) were used as a cell model to demonstrate the effects of STC-1 and STC-2 on cell proliferation, migration and tube formation. The involvement of some key angiogenic markers/regulators, like VE-cadherin, VEGF/VEGFR-signaling, eNOS and angiopoietins in STC-1 and/or STC-2 mediated angiogenic sprouting were investigated.

2. Materials and methods

2.1. Cell culture

The human umbilical vein endothelial cells (HUVEC, Clonetics San Diego, CA) were grown in M199 (Invitrogen) supplemented with 20% FBS (HyClone, Perbio), antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) (Invitrogen) 20 μg/ml endothelial cell growth supplement and 90 U/ml heparin (Sigma) in a humidified incubator at 37 °C with 5% CO_2.

2.2. Lentiviral STC-1/STC-2 expression constructs

The in-frame coding regions of human STC-1 and STC-2 without the stop codon, were PCR-amplified and ligated into pENTR™/SD/D-TOPO (Invitrogen) according to the manufacturer's instruction. The lentiviral STC-1 or STC-2 expression constructs were generated by LR recombination reaction between the entry clones and pLenti6.3/TO/V5/-DEST (Invitrogen) using Gateway® LR ClonaseTM II Plus Enzyme Mix (Invitrogen). The sequences of the STC-1 or STC-2 inserts were verified by DNA sequencing.

2.3. Lentiviral transduction of HUVEC

STC-1 and STC-2 lentivirus were generated by using ViraPower™ HiPerform™ T-RexTM Gateway® Expression System (Invitrogen) according to the manufacturer's instruction. HUVEC (1×10^6) were plated in 100 mm^2 dishes for overnight incubation, and were then transduced with 5 ml of a) pLenti (Ctrl), b) STC-1/pLenti- or c) STC-2/pLenti-virus containing medium with 6 μg/ml of polybrene® (Sigma). After 24 h incubation, the viral medium was replaced by M199. The overexpression of STC-1-V5 or STC-2-V5 in HUVEC cells were confirmed by western blotting using anti-STC-1, anti-STC-2 or anti-V5 antibody.

2.4. Proliferation assay

Cell growth was evaluated by DELFIA® Cell Proliferation kit (PerkinElmer). Lentiviral transduced HUVEC (8×10^3 per well for the control, STC-1-V5 or STC-2-V5) were plated in triplicate in 96-well dishes. After overnight incubation, the cells were incubated with BrdU for 12 h. Cell proliferation assay was carried out according to the manufacturer's instructions. Eu-fluorescence was measured by a multilabel reader VICTOR™X4 (PerkinElmer, Waltham, USA).

2.5. Gelatin zymography

One hundred g of serum-free conditioned media were loaded in a non-reducing SDS–PAGE gel containing 0.1% gelatin. The gel was washed with 0.1% Triton X-100 for 30 min, then incubated overnight at 37 °C in a developing buffer (50 mM Tris–HCl, 20 mM $CaCl_2$, pH 7.4) and visualized by staining with Coomassie Blue.

2.6. Cell migration assay

Lentiviral transduced HUVEC (1×10^4 per well for the control, STC-1-V5 or STC-2-V5) were seeded in 24-well Transwell inserts with 8 m pore size membrane (Costar, Corning) with serum free M199 medium. The medium with 5% serum as a chemo-attractant was added into the lower chamber. After 18 h of incubation, non-invading cells on the upper membrane were removed with cotton swabs. The invaded cells were fixed with ice-cold methanol for 10 min, and incubated for 15 min with a crystal violet solution. The total number of migrated cells were then photographed and counted under microscope. Antibody neutralizing cell migration assay was performed in the presence of specific goat anti-STC-1, anti-STC-2 or an isotype matched IgG antibody (10 μg/ml) (R&D).

2.7. Tube formation assay

Lentiviral transduced HUVEC (1×10^5 per well for the control, STC-1-V5 or STC-2-V5) were seeded in 96-well plates coated with growth factor-reduced matrigel (BD Bioscience). After 8 h incubation at 37 °C, the degree of tube formation and branch points of the cells were quantified by counting the number of tube-like structures in 6 randomly chosen fields for each well without overlap.

2.8. RNA extraction and real-time PCR

Cells were dissolved in TRIZOL reagent (GIBCO/BRL). Total RNA was extracted according to the manufacturer's instructions. The RNA A_{260}/A_{280} ratio was between 1.6 and 1.8. Reverse transcription was carried out using HC RNA-cDNA Master Mix (Applied Biosystems). Real time-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems). The primers were designed on the basis of the published sequences of human STC1 [TGAGGCGGAGCAGAA TGACT-forward and CAGGTGGAGTTTTCCAGGCAT-reverse] (Yeung and Wong, 2011), STC2 [GGTGGACAGAACCAAGCTCTC-forward and CGTTTGGGTGGCTCTTGCTA-reverse] (Law et al., 2008a), VEGF [CGAAACCATGAACTTTCTGC-forward and CCTCAGTGGGCACAC ACTCC-reverse] (Law et al., 2010), Ang-1 (NM_001146.3) [TGGC CCAGATACAGCA GAATG-forward and TGATTTAGTACCTGGGT CTCAACA], Ang-2 (NM_001147.2) [CAAATCAGGACACACCACGA ATG-forward and ATCAACGCTGCCATCCTCA C-reverse], eNOS (NM_000603.4) [CGGAGAATGGAG AGAGCTTTG-forward and AACT CTTGTGCTGTTCCGGC-reverse], actin [GACTACCTCATGAAGATCCTC ACC-forward and TCTCCTTAATGTCACGCACGATT-reverse]. Quantified standard and sample cDNA were analyzed. The amplification cycles were 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 10 s and 72 °C for 30 s using the ABI StepOne Real-time PCR System (Applied Biosystems). Following PCR amplification, the reaction products were resolved at 100 V on a 1% agarose gel with 0.5 μg/ml ethidium bromide. All glass- and plastic-ware were treated with diethyl pyrocarbonate and autoclaved.

2.9. Western blot analysis

The treated cells were washed with 2–3 changes of cold PBS. Adherent cells were scraped from the plastic surface and transferred to a microcentrifuge tube. The cells were pelleted and resuspended in a cold lysis buffer containing 250 mM Tris/HCl, pH 8.0, 1% NP-40 and 150 mM NaCl. After 10 min incubation on ice, the lysed cells were pelleted and supernatants were assayed for protein concentration (DC Protein Assay Kit II, Bio-Rad Pacific Ltd). Samples were subjected to electrophoresis and then were blotted

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