Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Rap1 promotes proliferation and migration of vascular smooth muscle cell via the ERK pathway

Qin Li, Yunfei Teng, Jian Wang, Miao Yu, Yiqing Li, Hong Zheng*

Department of Vascular Surgery, Union Hospital Affiliated with Tongji Medical College of Huazhong University of Science and Technology, China

ARTICLE INFO

Keywords:
Vascular smooth muscle cell
Rap1
Rap1-GAP
Proliferation
Migration

ABSTRACT

Background: Rap1 is involved in a multitude of cellular signal transduction pathways, which has extensively been linked to cell proliferation and migration. It has been shown to be important in the regulation of physiological and pathological processes. The present study aims to elucidate its detailed mechanistic in proliferation and migration.

Material/methods: Vascular smooth muscle cells (VSMCs) were transfected with pcDNA3.1(empty vector), pcDNA3.1 containing Myc-Tagged-Rap1V12 (Rap1V12) or pcDNA3.1 containing Flag-Tagged-Rap1GAP (Rap1GAP).The cells were presence or absence with 8CPT-2′OMe-cAMP or SDF-1 before transfection. The proliferation and migration were examined by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and transwell analysis, respectively. Afterwards, western blot was performed to detect the expression of ERK, phosphorylated-ERK, Rap1, Rap1GAP and Rap1GTP.

Results: The results showed that proliferation, migration and the expression of Rap1, Rap1GAP, p-EKR were boosted in treatment of Rap1V12-transfection. However, Rap1GAP presented the opposite effects. Subsequently, VSMCs were pretreatment with stimulators Rap1 guanine exchange factor (Rap1GEF), 8CPT-2′OMe-cAMP and stromal cell-derived factor 1 (SDF-1), then transfected with different vectors and the expression of Rap1, Rap1GAP and p-EKR were obviously decreased.

Conclusions: Taken together, these findings indicated for the first time that Rap1 was essential for the VSMCs in proliferation and migration by ERK signaling pathway.

1. Introduction

The small GTPase Ras-related protein 1 or Ras-proximate-1 (Rap1) is inactive in the GDP-bound form following interaction with a guanine exchange factor (GEF) and active in the GTP-bound form following interaction with a GTPase activating protein (GAP) [1]. The functions which was a molecular switch cycling between an inactive GDP bound form and an active GTP-bound form. It has been reported that Rap1 regulated cell adhesion dynamics and phagocytosis, especially by mediating the functions of integrins and cadherins. Rap1 signaling pathway contributed to cell invasion through physical pushing in various tumor cells [2–5]. In addition, research revealed that activation of Rap1 promoted melanoma cell proliferation and migration through the mitogen-activated protein kinase (MAPK) pathway and integrin activation. Therefor, this data indicated that Rap1 might act as an oncogenic role in melanoma [6]. The melanoma cells overexpression of Rap1GAP blocked Rap1 activation and extracellular signal-regulated kinase (ERK) phosphorylation, subsequently, it inhibited melanoma cell proliferation and survival, consequently decreased melanoma cell migration.

Vascular smooth muscle cells (VSMCs) were the main component of the artery's medial layer. These cells undergone contraction and regulated blood vessel tone and consequently blood flow and pressure [7]. Ras in coordinating signals in VSMCs to control proliferation and migration. However, the biochemical mechanism by which potentially mediated VSMCs which was not clear. The data suggested that CD98 heavy chain was required for VSMCs proliferation, and that its deficiency led to significantly reduced the presence of VSMCs in the neointima [8]. The two major phenotypes of VSMCs included fully differentiated, contractile cells responsible for vasodilation and vasoconstriction, and migratory, proliferative cells that were activated during growth or injury [9].

Previous study had found that Rap1 played a critical role in human umbilical vein endothelial cells (HUVECs). A novel insight into the mechanism by which Rap1GAP/Rap1 regulated proliferation and migration of HUVECs via ERK and Akt pathways were elucidated. These

* Corresponding author at: Department of Vascular Surgery, Union Hospital Affiliated with Tongji Medical College of Huazhong University of Science and Technology, No. 1277, Jiefang avenue, Jianghan District, Wuhan, China.
E-mail address: 1651283602@qq.com (H. Zheng).

https://doi.org/10.1016/j.prp.2018.04.007
Received 30 January 2018; Received in revised form 29 March 2018; Accepted 13 April 2018

results were interesting, so how does Rap1 regulated the VSMCs proliferation and migration, whether Rap1 regulated VSMCs in the same way remains unclear. This study was to elucidate a detailed mechanistic process of the Rap1-regulated VSMCs proliferation and migration.

2. Materials and methods

2.1. Isolation and culture of rat VSMCs

Experiments were performed according to institutional guidelines and approved by Ethical Committee of Tongji Medical College of Huazhong University of Science and Technology. Wistar rats, about 250 g body weight were killed by CO2 anesthesia and decapitated the fetuses were removed and kept on ice in phosphate-buffered saline (PBS) at pH 7.4. The following micropreparation steps were performed under a dissection microscope. The pericardium was opened and the great vessels was rinsed with PBS (4 °C).The prepared tissues were dissociated enzymatically at 37 °C overnight in an incubation solution of 2 mL medium containing the following: collagenase type II 2 mg/mL (Sigma, USA), elastase 0.2 mg/mL (Roche, USA), trypsin inhibitor 0.5 mg/mL, BSA 2.0 mg/mL, and DNAase type I 0.1 mg/m (Sigma, USA). The digestion was stopped by adding 2 mL of VSMCs medium. Then the cell suspension was centrifuged for 5 min (800 rpm, 4 °C), and the pellet was resuspended in VSMCs medium. Finally, the cell suspension was filtered through a 20 um metal mesh once, and cells were disseminated into 250 mL cell culture flasks. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2.

2.2. Plasmids

pcDNA3.1 empty vector (pcDNA), pcDNA3.1 containing Flag-Tagged- Rap1GAP and pcDNA3.1 containing Myc-Tagged-Rap1V12 were kindly provided by two professors. The Rap1V12 and Rap1GAP were amplified from cDNA constructs with Phusion high fidelity DNA polymerase subcloned into pcDNA3.1 (−) with Myc or Flag tag at its 5′ end of cloning site so that expressed protein has a tag at its N-terminal end.

2.3. Cell transfection and treatment

When the VSMCs were grown about 80% confluence. The cells were transfected with pcDNA3.1 empty vector alone (pcDNA), pcDNA3.1 containing Flag-Tagged-Rap1GAP (Rap1GAP) (generous gift of Dr. P. Stork, Oregon Health Sciences University, USA) or Myc-Tagged-Rap1V12 (Rap1V12) (kindly provided by Dr. H. Zhao of Washington University, USA) using Fusion 6 (Roche, USA) for 48 h according to the manufacturer's instructions. The transfected clones were selected by using G418-containing media (600 μg/mL) (Sigma, USA). Clones expressing higher levels of Flag protein or Myc protein were propagated and maintained in the same selective medium in 37 °C 5% CO_2 at for the subsequent experiments. The partial VSMCs were treated or untreated with stimulators of 8CPT-2′OMe-cAMP (50 μmol/L) or stromal cell-derived factor 1 (SDF-1, 30 μmol/L), follow transfected with different vectors which were mentioned above, respectively.

2.4. Cell viability

Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Boster, Wuhan, China). The VSMCs were transfected with pcDNA3.1 empty vector (as control group) or Rap1V12, about 4×10^4/well were seeded into 96-well plates and cultured in incubator in 37 °C 5% CO_2, respectively. After culture for 48 h, the supernatant were removed and 20 μL of MTT reagent were added in each well of a 96-well plate and incubated for 4 h. Then the supernatant was removed and the cells were treated with 100 uL/well formazan solution for 10 min. After that the absorbance was detected at 570 nm. Cell viability = (the average optical density (OD) value of experimental group/the average OD value of control group) × 100%. The experiments were performed in triplicate. Likewise, cell viability was detected in VSMCs transfected, respectively, with vector pcDNA3.1 containing Flag-Tagged-Rap1GAP (Rap1GAP) or pcDNA3.1 empty vector pretreatment absence or presence 8CPT-2′OMe-cAMP (50 μmol/L) or SDF-1 (30 μmol/L), respectively.

2.5. Transwell assay

The aim of transwell assay was to analyze the migration of the pcDNA3.1 empty vector or Rap1V12 transfected with VSMCs cells. The transwell experiment was conducted by cell culture inserts with 8.0-μm pores (Corning Costar, USA), which were placed in a 24-well plate system. To test the migration ability, 5×10^4 cells were seeded into the upper chamber with 200 μL FBS-free DMEM medium, and 800 μL medium containing 10% FBS was added into the lower well. After cells migrating to the bottom of the chamber membrane were fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) nuclear-stained and the number of cells was counted in a constant number of sequential random fieldsusing fluorescent microscopy. Migration was similarly evaluated in VSMCs transfected with vector pcDNA3.1 containing Flag-Tagged-Rap1GAP, respectively. On the other hand, the partial cells were pretreated with or without 8CPT-2′OMe-cAMP (50 μmol/L) or SDF-1 (30 μmol/L), stimulation for 12 h then transfected with vectors which mentioned above to assess the migration of VSMCs by the same way.

2.6. Western blotting

On the one hand, the VSMCs were cultured in triplicate 6-well plates transfected with pcDNA3.1 empty vector (control) alone, pcDNA3.1 containing Rap1GAP or Rap1V12, respectively. On the other hand, the partial cells were pretreated with 8CPT-2′OMe-cAMP (50 μmol/L) or SDF-1 (30 μmol/L), then transfected with pcDNA3.1 empty vector (control), pcDNA3.1 containing Rap1GAP or Rap1V12, respectively. And the other part of VSMCs were treated with SDF-1(30 umol/L)under different time points from 0 to 20 min, Different treatments above cells were lysed with radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Jiangsu, China) containing phenylmethanesulfonyl fluoride (PMSF). The total protein concentrations were determined using the bicinchoninic acid (BCA) assay. The proteins were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk for 4 h at room temperature and incubated with a primary antibody (anti-β-actin, anti-Rap1-GTP, anti- Rap1-GAP, anti-p-ERK, anti-ERK, anti-Rap1; Cell Signaling, Danvers, MA, USA) at a 1:5000 dilution at 37 °C for 24 h. The membranes were then washed three times with Tris-buffered saline/Tween (TBST) buffer, followed by incubation with a secondary antibody (goat anti-mouse IgG; Cell Signaling, Danvers, MA, USA) used at a dilution of 1:1000 at 37 °C for 2 h. An enhanced chemiluminescence (ECL) reagent was used to detect the protein bands by exposing the blots to autoradiographic film. The bands were quantified in ImageJ software by using β −actin as a loading control. This test was repeated for at least three times.

2.7. Statistical analysis

Experiments were independently conducted VSMCs, each performed in triplicate or duplicate, respectively. Comparisons between groups were analyzed by *t*-test and Bonferroni's post hoc ANOVA test employed for experiments with more than 2 groups. Data are presented as the mean ± SD of three independent experiments. A P value less than 0.05 was considered to be statistically different.