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Inhibition of farnesyltransferase reduces angiogenesis by interrupting endothelial cell migration

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ABSTRACT

Inhibitors of farnesyltransferase (FTI) have been developed for cancer treatment for more than a decade. Aside from being a therapeutic target in tumor cells, little is known about the role of farnesyltransferase (FTase) in other physiological processes. In this study, we revealed the involvement of FTase in angiogenesis and showed that FTI inhibited angiogenesis by directly acting on endothelial cells. Inhibition of FTase interrupted cell migration in vitro and in vivo. In addition, we found that FTase was important for cell polarization, cell spreading and pseudopodia formation. We also found that FTase interacted with microtubule end binding protein 1 (EB1) and that this interaction was critical for the localization of EB1 to microtubule tips. Our findings thus offer novel insight into the functions of FTase in endothelial cells and provide valuable information for the use of FTI in cancer therapy.

1. Introduction

Farnesyltransferase (FTase) catalyzes the covalent attachment of a farnesyl group to the CAAX (C is cysteine, A is an aliphatic amino acid, and X is variable) motif at the carboxyl terminus of a protein [1,2]. Addition of this 15-carbon isoprenoid alters the properties of many important cellular proteins including nuclear lamins, kinetochore proteins CENP-E and CENP-F, as well as members of the Ras, Rho and RheB families [1,3,4]. Functional FTase is a dimer with its β subunit harboring the active site. Farnesylation has been shown to be critical for the proper localization of Ras and Rho to cell membrane by conferring them lipid-binding ability, which is prerequisite for their transforming power [4]. Linkage between farnesylation and activation of these oncogenic proteins hence prompted extensive studies focusing on the role of FTase in malignant cells, and attempts to use inhibitors of farnesyltransferase (FTI) for cancer treatment have made some progress in the last decade [1,3,5,6]. Despite the potency of FTI in reducing proliferation and inducing apoptosis of cancer cell lines, clinical trials showed mixed responses, warranting detailed characterization of FTase in alternative processes of tumor development and in other cell types within the tumor microenvironment [5–9]. Investigations outside of the cancer context are providing insights into the functions of FTase in more physiological events [4,10,11], but molecular mechanisms independent of oncogenic Ras are still largely unknown.

Angiogenesis is a multistep process by which the existing vascular network is elaborated by formation of new blood vessels from pre-existing ones [12–15]. Angiogenesis holds a fundamental role in development and also functions in other physiological settings. Pathological angiogenesis, however, contributes to tumor growth and metastasis by providing nutrients and oxygen and serving as ducts for tumor cells [12,14,15]. Failure of or insufficient angiogenesis results in ischemic chronic wounds, while excess angiogenesis leads to age-related macular degeneration, demonstrating the importance of balanced angiogenesis for homeostasis [12,15]. FTI has been shown to be anti-angiogenic in tumor xenograft assays, due to its role in diminishing the release of angiogenic factors by targeting Ras-related cascades in tumor cells [1,3,4]. Emerging evidence suggests a broader effect of FTI in angiogenesis, pointing to the involvement of FTase in multiple endothelial functions.

In both normal and pathological angiogenesis, vascular endothelial cells adopt conserved mechanisms to migrate in response to angiogenic signals [12,16]. During migration, endothelial cells maintain a polarized morphology and extend multiple protrusions in probing the surrounding area [13,16–18]. Accurate interpretation of migration cues reflects the angiogenic potential of endothelial cells, which results from the dynamics of cytoskeleton and requires the cooperation between the cytoskeleton and associated proteins [13,16,18]. Microtubule end-binding protein (EB1) is a well established microtubule associated protein (MAP) which controls microtubule dynamics and plays an important role in the formation of polarized morphology and the motility of cells

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[18,19]. FTase has also been found to bind directly to microtubules [20], but the effect of FTase on microtubule dynamics remains elusive.

In this study, we found that FTase was critically involved in angiogenesis by regulating endothelial cell migration. Detailed analysis revealed the importance of FTase in cell polarity, cell spreading and pseudopodia formation. Moreover, we found that FTase interacted with EB1 and that inhibition of FTase interrupted the proper localization of EB1 to microtubule tips. Our results thus demonstrate the role of FTase in non-transforming cells and offer novel insight into the pharmacology of FTI in cancer treatment.

2. Materials and methods

2.1. Materials

The farnesyltransferase inhibitor SCH66336 and an inactive isomer of SCH66337, were from Schering-Plough (NY, USA). Matrigel was purchased from BD Biosciences (NY, USA). Antibodies against α-tubulin, γ-tubulin, actin and EB1 were obtained from Sigma–Aldrich (MO, USA). Small interfering RNAs (siRNA) targeting FTase were synthesized from Ribobio (Guangzhou, China).

2.2. Cell culture and transfection

Human umbilical vascular endothelial cells (HUVECs) were routinely maintained using RPMI1640 with 10% fetal bovine serum, in standard humidified cell incubator with 5% CO_2 at 37 °C. Cell transfection was performed with Lipfectamine 2000 reagent (Invitrogen, CA, USA).

2.3. RT-PCR

Total RNA was extracted from cells by TRIzol Reagent from Invitrogen (CA, USA). 2 μg RNA was used from each sample to perform cDNA synthesis with Reverse Transcriptase M-MLV from Promega (WI, USA). RT-PCR was then carried out using same amount of cDNA under thermal conditions as follows: 94 °C 5 min, 25 cycles of 94 °C 30 s, 56 °C 30 s and 72 °C 45 s, and 72 °C 5 min. PCR product was resolved by agrose gel and visualized under UV illumination. GAPDH was used as internal control.

2.4. Wound healing assay

Wound healing assay was performed as described before [13]. After treatment with FTI or transfection with siRNAs, HUVECs were allowed to reach confluence on 24-well plate. A pipette tip was used to create wound, with cell debris removed by several washes of PBS. Culture medium was added afterwards and the extent of subsequent wound healing recorded by imaging in 24 h. This assay was repeated 3 times and the percentage of wound closure was analyzed with ImageJ (NIH).

2.5. Tube formation assay and spheroid sprouting assay

Tube formation assay was performed as previously described [13,18]. HUVECs treated with FTI or transfected with siRNAs were resuspended and plated on 6-well plate precoated with matrigel. This assay was repeated 3 times. Images were taken in 6 and 24 h and analyzed with ImageJ. To make spheroids, HUVECs were seeded into round-bottom 96-well plate with culture medium containing 0.2% carboxymethylcellulose for 8 h. Spheroids of HUVECs were embedded into collagen afterwards and induced to form sprouts under normal culture condition. This assay was repeated 3 times. Images were taken and sprouts analyzed with ImageJ (NIH).

2.6. Matrigel plug assay

300 μL matrigel mixed with FTI or PBS was injected subcutaneously into 4–5 weeks BALB/c mice, and 3 mice were used in each group. The matrigel plug was recovered in 14 days and processed in accordance with standard histological procedures. Haematoxylin eosin (H&E) staining was performed to reveal the extent of cell migration inside matrigel. The use of mice was approved by the Animal Care and Use Committee of Nankai University.

2.7. Cell polarization assay

Monolayer of HUVECs was scratched with pipette tip to stimulate directed cell migration, with immunostaining of α-tubulin and γ-tubulin performed to visualize the organization of microtubules and the position of microtubule organizing center (MTOC). 4,6-Diamidino-2-phenylindole (DAPI) was used for DNA staining. Border cells with MTOC situated between the nucleus and the leading edge were taken as polarized.

2.8. Cell spreading assay

HUVECs treated with FTI or transfected with siRNAs were resuspended from dishes and re-seeded on coverslips with normal medium. Cells were fixed after 30 min with 4% paraformaldehyde and stained for actin to better visualize pseudopodia. This assay was repeated 3 times.

2.9. Immunofluorescent microscopy

Cells were fixed with 4% paraformaldehyde on coverslips and blocked with 2% bovine serum albumin. Primary and secondary antibodies were diluted and applied according to the instructions as well as previous knowledge. DAPI was used for DNA staining at the end of incubation and coverslips mounted with 90% glycerol. Images were taken with a fluorescence microscope and analyzed with ImageJ.

2.10. GST pull-down and western blotting

GST pull-down was performed as previously described [21,22]. Purified GST-EB1 was immobilized on glutathione Sepharose beads, which were incubated with lysate from control cells or cells overexpressing FTβ at 4 °C for 2 h. The beads were washed afterwards, proteins released and resolved by SDS-PAGE. Resolved proteins were then transferred onto PVDF membranes by electricity in accordance with standard protocols. After blocking with fat free milk, membranes were probed with primary and horseradish peroxidase-conjugated secondary antibodies. Band visualization was achieved by applying chemiluminescent substrate (Pierce, IL, USA).

2.11. Statistics

Analysis of differences between groups was performed using Student's *t*-test. Graphs were drawn and P values calculated using GraphPad Prism 5.

3. Results

3.1. FTase is essential for angiogenesis

The effect of FTase on angiogenesis was studied using HUVECs. Two different siRNAs were utilized, which reduced the transcription level of FTase to 38.6% and 19.5% of control group, respectively (Fig. 1A and B). When plated on matrigel, HUVECs began to form