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ORIGINAL ARTICLE

Lipoamide Inhibits NF1 Deficiency-induced Epithelial-Mesenchymal Transition in Murine Schwann Cells☆

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Background and Aims. Neurofibromatosis type I (NF1) is one of the most common neurocutaneous syndromes characterized by development of adult neurofibromas which is mainly made up of Schwann cells. The disease is generally accepted to be caused by inactivation mutation of *Nf1* gene. And *Nf1* deficiency had been reported to lead to ROS overproduction and epithelial-mesenchymal transition (EMT) phenotype. This study was designed to investigate whether excessive ROS conferred to *Nf1* deficiency-induced EMT in Schwann cells.

Methods. Colony formation, wound healing assay and transwell assay was used to evaluate the effects of stable *Nf1* knockdown in SW10 Schwann cells. Western blot and ROS assay was conducted to explore the molecular mechanisms of *Nf1* inactivation in tumorigenesis. Animal experiments were performed to assess the inhibitory effects of lipoamide, which is the neutral amide of α-lipoic acid and functions as a potent antioxidant to scavenge ROS, on *Nf1*-deficiency tumor growth *in vivo*.

Results. *Nf1* knockdown enhanced the cellular capacities of proliferation, migration and invasion, promoted ROS generation, decreased the expression of epithelial surface marker E-cadherin, and up-regulated several EMT-associated molecules in Schwann cells. Moreover, lipoamide dose-dependently inhibited not only *Nf1* deficiency-induced EMT but also spontaneous EMT. Furthermore, lipoamide markedly suppresses tumor growth in a mouse model of NF1-associated neurofibroma.

Conclusions. Our results clearly reveal that ROS overproduction is responsible for *Nf1* deficiency-induced EMT and plays a crucial role in NF1 tumor growth. The findings presented herein shed light on the potential of antioxidant therapy to prevent the progression of NF1-associated neurofibroma.

Key Words: Neurofibromatosis type I, EMT, ROS, Schwann cell, Lipoamide.

Introduction

Neurofibromatosis type I (NF1) is a multi-system disorder resulting from the prosoplasia of neural crest cells, with a morbidity of 1/3500 (1,2). It has been accepted that inactive mutation of *Neurofibromin 1* (*Nf1*), a gene located in 17q11.2 (3), is responsible for this disorder and can be detected in more than 95% of the NF1 patients (4,5). Moreover, accumulating evidences demonstrate that *Nf1* act as a tumor suppressor gene, and its mutations are presented in a large variety of non-*Nf1*-associated sporadic cancers, including melanoma, breast cancer, ovarian cancer, lung cancer, glioblastoma, urine cancer and acute myeloid leukemia (6).

Neurofibromin, protein encoded by *Nf1* gene, is a GTPase-activating protein which can bind to GTP-bound RAS to enhance its intrinsic GTPase activity and hence

Conflict of Interest: There is no conflict of interest.

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functions as a Ras off signal (7). Therefore, inactivation of neurofibromin leads to an augmentation of Ras signaling pathway, and consequently results in enhanced reactive oxygen species (ROS) production (8). In addition, loss of neurofibromin in *Drosophila melanogaster* was found to promote ROS generation in a Ras-independent manner (9). Recent evidences showed that in human Schwann cells, human epithelial-like breast cancer cells and mouse epicardial-derived cells the decreased expression of neurofibromin promotes epithelial-mesenchymal transition (EMT), a process which plays crucial roles in tumor progression and metastasis (10,11). Although it is well-documented that Ras activation is involved in EMT induction, the role of ROS in EMT induction is contradictive. It is prevailing that ROS plays a crucial role in EMT induction (12−14), excessive ROS was recently reported to inhibit EMT occurrence in prostate cancer cells (15), and decreasing ROS generation by epigenetic silencing of fructose-1, 6-biophosphatase was found to promote EMT phenotype in basal-like breast cancer (16,17). Furthermore, TGF-β-induced EMT-like phenomenon was shown to be associated with an increased ROS level in liver cancer cells (18). In addition, diallyl trisulfide treatment was found to inhibit EMT progress in MDA-MB 231 cells through increasing ROS (19). Downregulation of GRIM-19 and NDUFS3, two subunits of mitochondrial complex I, decreased ROS generation and led to EMT phenotype in HeLa cells (20). In conclusion, ROS may play a dual effect in regulating EMT depend on cellular context.

In this study, we first modeled NF1 *in vitro* by knockdown *Nf1* expression in murine Schwann cells and evaluated the effect of *Nf1* deficiency on ROS generation and EMT progress. We also investigated the impact of lipoamide, a ROS scavenger, on *Nf1* deficiency-induced EMT to elucidate the role of ROS overproduction in EMT induction in Schwann cells. Finally, we evaluated the impact of lipoamide treatment on tumor growth by using an *in vivo* model of NF1-associated neurofibroma.

Method and Materials

Cell Culture and Stable RNA Interference

Murine SW10 Schwann cell line was maintained in DMEM supplemented with 10% (V/V) fetal bovine medium at 37°C in a fully humid atmosphere containing 5% CO_2. Two recombinant replication-defective lentiviruses harboring expression cassettes coding for either *Nf1*-specific short hairpin RNA (shRNA) or non-silencing shRNA control were used to infect the SW10 cells and the transfected cells were named Nf^{kd} and $Nf1^{nd}$ cells, respectively. And the knockdown efficiency was determined by both quantitative RT-PCR (qRT-PCR) and western blotting.

RNA Extraction and qRT-PCR

Nf^{kd} and $Nf1^{nd}$ cells were collected and total RNA were extracted using Fastagen RNA fast 200 following the manufacturer's protocol. Then the cDNA was synthesized according to the manufacturer's protocol of TaKaRa PrimeScript™ RT Master Mix. The level of *Nf1* mRNA was determined using the reaction system of TaKaRa SYBR® premix ExTaq II with primers 5′-GTGCTGTTTGTGCTGAGCTGTGAA-3' (forward) and 5′-TCTATTGAACTGCCCATACCCGCA-3' (reverse). The mRNA expression of *Nf1* was normalized to GAPDH which served as an internal control. The primers used for GAPDH detection were 5′-ATGGGGAAGGTGAAGGTCGG-3' (forward) and 5′-GACGGTGCCATGGAATTTGC-3' (reverse). The data of the expression levels of mRNA was analyzed by delta Ct.

Cell Proliferation Assays

Nf^{kd} and $Nf1^{nd}$ SW10 cells (1000/well) were cultured in 6 well plates in 2 mL DMEM complete medium with 10% FBS. The medium was refreshed every 24 h. After 7 d of culture, the cells were fixed with methanol and stained with 0.5% crystal violet, and then colonies (more than 50 cells per colony) were counted. Each experiment was performed in triplicate. The number of colonies was analyzed and expressed as the average number of colonies.

Cell Migration Assay

Wound healing assay was performed to test the cells' ability of migration. Nf^{kd} and $Nf1^{nd}$ SW10 cells were cultured in 6 well plates overnight. The wounds were created by 200 μL tip. Then, the cells were washed with cold sterile PBS (pH 7.4) for three times and were cultured in 2 mL serum-free medium. Images were taken randomly for five fields of each kind of cells at 0, 24 and 48 h under microscope. (Magnification for each set:×100).

Cell Invasion Assay

Cell invasion assays were assessed by 8.0 μm pore size transwell chambers pre-coated with Matrigel (5 × dilution; 50 μL/well; BD Bioscience, NJ) stewing overnight at 37°C. Nf^{kd} and $Nf1^{nd}$ SW10 cells (1×10^5) were seeded in the upper chamber in 200 μL of serum-free medium. DMEM with 10% FBS (0.6 mL) was added to the lower chamber. After 48 h, non-invading cells on the upper surface of the membrane were wiped out by cotton swab and invading cells were fixed in 100% methanol and stained with crystal violet solution. Images were taken randomly for five fields of each membrane. The average numbers of the invading cells per microscopic field over five fields were analyzed. (Magnification for each set:×200).